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THE UTILIZATION OF GALACTOSE FOLLOWING COMPLETE REMOVAL OF THE LIVER

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One of the most important functions of the liver is the maintenance of the normal level of sugar in the blood. Except for the periods when glucose is added to the blood by alimentation, the liver is constantly adding glucose to the blood at the expense of its own glucose and glycogen. The liver is able to replenish its supply of glucose and glycogen by the conversion of other substances to glucose. The conversion of some of these substances appears to be specific for the liver since it is not accomplished by the other tissues in the absence of this organ. Amino acids (1) are not diaminized or converted to glucose in the absence of the liver. Lactic acid may be converted to muscle glycogen but is not utilized to increase the sugar content of the blood except by the liver. Fructose, however, may be converted to glucose in the absence of the liver. Bollman and Mann (2) found definite elevation of the glucose content of the blood of hepatectomized dogs and relief of symptoms of hypoglycemia following the administration of fructose to these animals. This conversion of fructose to glucose appeared to be accomplished by the intestine since no increase of blood glucose and no alteration of symptoms of hypoglycemia occurred when fructose was administered to animals from which both liver and intestines had been removed. Definite elevation of the blood glucose was also demonstrated following removal of the intestines of animals of which the liver had not been disturbed. Apparently both the liver and the intestines are able to convert fructose to glucose. The glycogen content of the muscles could be increased by administration of fructose in the absence of the liver but only under conditions that also increased the glucose of the blood. It could not be definitely established that the fructose was directly converted to muscle glycogen but this appeared unlikely since increases of glycogen were not obtained from administration of fructose to animals of which both liver and intestines had been removed.

The metabolism of galactose has been reviewed recently by Shay, Schloss and Bell (3). They concluded that galactose is an ideal sugar for liver function tests since it is readily absorbed from the intestine and is readily excreted by the kidneys even in the presence of severe renal damage. Cori (4) found that galactose increased the glycogen content of the liver much less than did equal amounts of glucose or fructose. Cori and Cori (5, 6) observed a greater utilization of galactose with decreased rates of absorption when the galactose was given enterally. The total amount utilized, however, increased with increased absorption so that as much as 52 mgm. could be utilized for each 100 grams rat per hour. Wierzuchowski and his collaborators (7, 8) used continuous intravenous injection of galactose, 2 grams for each kilogram of body weight each hour for three hour periods. Under these conditions their dogs excreted about 70 per cent of the injected galactose and galactose remained in the blood for nine hours after injection. The same animals excreted only 10 per cent of glucose or fructose when equal amounts of these sugars had been given in the same way. Injections of insulin did not greatly alter the excretion of glucose or fructose but slightly reduced the amount of galactose excreted in the urine. No significant changes were observed in the blood or urine glucose following the injection of fructose or galactose. Studies of respiratory metabolism indicated that the portion of retained galactose which was oxidized was much larger than was the portion of glucose or of fructose which underwent oxidation. Less storage or conversion of galactose to glycogen occurred than was the case with glucose or fructose.

Methods. The rate of disappearance of galactose from the blood after injection of galactose, and the amount of this sugar excreted in the urine, were determined on dogs under three physiologic conditions: normality, following complete removal of the liver, and after the development of extreme degrees of experimental cirrhosis of the liver. All animals were accustomed to the laboratory procedures and variations in their blood sugar were not attributable to the handling incident to venipuncture or catheterization. All animals had been maintained on an adequate mixed diet for several weeks and were used each time after a fast of eighteen hours.

The method of administration of galactose which we found to be most suited for this type of experiment was the intravenous administration of 500 mgm. of galactose for each kilogram of body weight. Oral administration was found to give widely divergent results on normal animals. The dosage of 500 mgm. of galactose for each kilogram of body weight was adopted because smaller doses disappeared rapidly from the blood and the amount of galactose excreted in the urine was variable. Larger doses remained in the blood longer, and larger amounts of galactose were excreted in the urine so that the amount retained (and subjected to hepatic activity) was reduced in terms of percentage of the amount administered.

Galactose, 500 mgm. for each kilogram of body weight, showed the greatest difference in utilization of this sugar by the normal animal and by the animal totally deprived of hepatic tissue.

Specimens of blood were obtained at appropriate intervals by puncture of the jugular vein. Urine was obtained at timed intervals by catheterization.

Glucose and galactose in the blood were determined in the protein-free filtrates of unlaked blood prepared according to the method of Folin. For this purpose the freshly drawn blood, without an anticoagulant, was measured out immediately and delivered into a previously measured volume of cooled sulphate-tungstate reagent. The samples were stored in the refrigerator until the end of the experiment; then all were acidified, centrifuged, and filtered. Control experiments showed no changes to have occurred in the glucose or galactose content of the blood thus preserved in the refrigerator. The reducing power of the filtrates was determined by means of a Shaffer-Hartman alkaline copper solution similar in composition to the modified reagent devised by Somogyi (9), except that in accordance with the suggestion of DeLong (10), potassium iodide was omitted. This was added later, in each individual determination, just before the titration with 0.01 N thiosulphate. For the determination of galactose plus nonsugar reducing substances, the filtrates were subjected to fermentation with washed yeast according to Somogyi's (11) method as slightly modified by Spannuth and Power (12). Under the conditions employed glucose is removed quantitatively, while galactose, according to Cave (13), is unaffected. The difference in the reducing power before and after fermentation is therefore calculated as glucose, whereas the reduction after fermentation, corrected for the non-sugar reducing substances as determined on the control samples of blood before the administration of galactose, is calculated as galactose. In each case the titration values corresponding to glucose and galactose were converted to concentration values by means of curves previously constructed with the aid of pure glucose and galactose solutions respectively. In agreement with recent work it was found that the reducing power of galactose with the particular copper reagent used was about 80 per cent of that of glucose. Likewise, it was found that in mixtures of glucose and galactose, there was no detectable influence of either sugar on the reducing power of the other.

Urinary sugar was determined in a similar manner to that in the blood, by the Shaffer-Hartman method. The yeast treatment of the urine was omitted, however, in many instances because the high concentration of galactose and the absence of glucose from the urine allowed sufficient dilution so that the correction for the reducing substances in the urine became negligible.

Complete hepatectomy was performed by the method of Mann (14), in

which two preliminary operations are utilized. The first establishes an anastomosis of the portal vein and the vena cava; the vena cava is ligated above the stoma just below the liver. After several weeks an extensive collateral circulation of the portal and caval regions has developed. The second operation, ligation of the portal vein, serves to test the adequacy of the collateral circulation to return the blood to the heart. If this is inadequate death will follow in a few hours from portal stasis which would be attributed to the absence of the liver had that organ been removed at this time. After a week or two the liver may be removed. This is rapidly accomplished and the animal recovers from the ether anesthesia and appears normal. After several hours symptoms of hypoglycemia develop which may be entirely dispelled by the administration of glucose.

Partial impairment of hepatic function of a number of dogs was produced by repeated administration of carbon-tetrachloride (15). From 5 to 10 cc. of carbon-tetrachloride were administered by stomach tube three or four times each week. This produces a series of acute degenerative changes in the liver with subsequent adenomatous proliferation of hepatic cells and the formation of cicatricial tissue in the liver. Some of the animals used in this series had been under treatment for almost three years and all had moderate degrees of bilirubinemia and definitely retained bromsulphalein. Other animals were more acutely intoxicated with carbon-tetrachloride and the degree of bilirubinemia was used as an index of hepatic damage which was confirmed by histologic examination of the liver at the end of the experiment.

Results. Following the intravenous injection of 500 mgm. of galactose for each kilogram of body weight into normal dogs, the galactose content of the blood progressively diminished so that not more than traces remained in the blood after two hours. The urinary excretion of galactose was practically completed at this time. The amount of galactose which appeared in the urine of normal dogs varied from 50 to 150 mgm. per each 500 mgm. injected under these conditions. No significant changes were observed in the glucose content of the blood or urine. In the absence of renal excretion (nephrectomized dogs) the disappearance of the injected galactose from the blood was somewhat more prolonged, but the galactose usually was found to have completely disappeared within three hours.

After total removal of the liver, intravenously injected galactose, 500 mgm. for each kilogram of body weight, progressively disappeared from the blood so that only traces remained after three hours (fig. 1). The curve of the blood galactose was only slightly higher than that found when a normal dog was used. The complete urinary excretion of galactose ranged from 250 to 310 mgm. for each 500 mgm. injected in the different experiments. In the absence of renal excretion (nephrectomized-hepatectomized dogs) the galactose remained in the blood longer, so that four or

five hours after injection of galactose only traces were found in the blood (fig. 2). But it was apparent that the galactose was gradually being withdrawn from the blood.

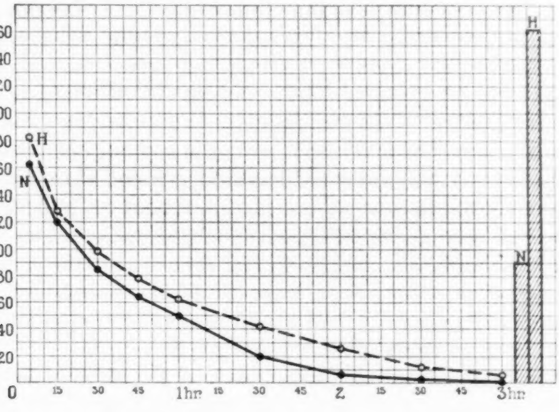

Fig. 1. Curves showing the clearance of galactose from the blood following the intravenous injection of 500 mgm. of galactose for each kilogram of body weight in the normal, *N*, and hepatectomized, *H*, dog. The curves are expressed in milligrams of galactose for each 100 cc. blood. The rectangles indicate the total amount of galactose excreted in the urine; shown as milligrams for each kilogram of body weight so that 500 would represent complete recovery of galactose.

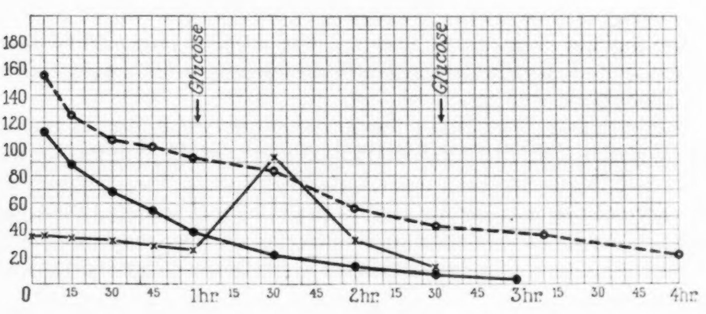

Fig. 2. Curves showing the clearance of galactose from the blood of nephrectomized dogs following the intravenous injection of 500 mgm. of galactose for each kilogram of body weight. The broken line is the blood galactose curve of a nephrectomized, hepatectomized dog; the solid line that of a nephrectomized dog in which the liver was not disturbed. The solid line marked *X* shows the glucose content of the blood of the hepatectomized nephrectomized dog.

The glucose content of the blood of hepatectomized dogs continued to decrease after administration of galactose, and symptoms of hypoglycemia developed in relationship to the glucose content of the blood and were independent of the galactose content. In most experiments it could not be shown that there was any change in the usual rate of decrease of the glucose content of the blood. In a few experiments some delay appeared and minor increases of 1 or 2 mgm. of glucose for each 100 cc. of blood were noted. This will be discussed later. No significant changes were found in the glycogen content of the muscles of the hepatectomized dog which had received galactose. The decrease in glycogen content of the muscles that follows hepatectomy was present after administration of galactose. However, most of the animals would not have survived the hypoglycemia unless glucose had been given; so the survival time without glucose was too short to allow significant changes in the glycogen of the muscles to be demonstrated, even if muscle glycogen could be formed from galactose. Changes are seldom demonstrated within the first six hours after hepatectomy, when large amounts of glucose are given.

The disappearance of injected galactose from the blood of animals which had marked experimental cirrhosis was similar to that of normal animals. The urinary excretion of galactose was usually found to be from 100 mgm. to 200 mgm. for each 500 mgm. injected. If acute degenerative changes were present in the liver, as occurs for several days following administration of carbon-tetrachloride, more galactose appeared in the urine; 150 mgm. to 250 mgm. for each 500 mgm. injected were recovered. Similar results were obtained with hepatic injury from carbon-tetrachloride of animals that previously had been normal. Histologic examination of sections of liver removed immediately following the experiment indicated that the amount of galactose excreted was roughly proportional to the degree of acute hepatic injury and bore no relation to the amount of cicatricial tissue present in the liver.

Galactose was injected into a few animals from which part of the liver had been removed (fig. 3). From 50 to 70 per cent of the liver was removed after an Eck fistula had been made. Regeneration of liver such as occurs in the normal dog was not present. These animals excreted essentially the same amount of galactose after operation as they did before. The liver that remained, however, appeared approximately normal on histologic examination.

Discussion. The normal dog utilizes galactose much more slowly than it utilizes glucose or fructose. In the absence of the liver, utilization is still further retarded and a much larger portion of the galactose which has been administered is excreted in the urine. It should be noted that not all of the galactose administered is recovered in the urine. The retained galactose disappears completely from the blood and it is extremely unlikely that

it is stored elsewhere in the body as galactose. No appreciable galactose depots have been demonstrated, and after repeated administrations of galactose to liverless animals, there is similar disappearance of galactose, with no evidence of storage of galactose as such.

In our experiments we have been unable to determine the fate of the galactose which is apparently utilized without the intervention of the liver. Three possibilities present themselves. The galactose may be oxidized

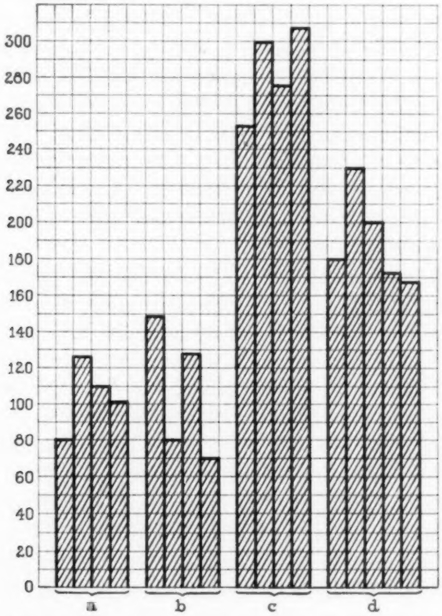

Fig. 3. Rectangles representing the excretion of galactose following administration of 500 mgm. of galactose for each kilogram of body weight. The height of the rectangle indicates the number of milligrams of galactose in the urine for each kilogram of body weight. Five hundred would represent complete recovery of the galactose. *a*, normal dogs, *b*, dogs with more than 50 per cent of the liver removed, *c*, dogs with the liver completely removed, and *d*, dogs with acute hepatic degeneration following carbon-tetrachloride.

directly, converted to glucose, or converted to glycogen. Direct oxidation of galactose might be of a non-specific nature; its fate might be that of a foreign substance introduced into the body, and it might be destroyed in a manner comparable to the destruction of alcohol, with little direct effect on the normal carbohydrate metabolism of the tissues. If the galactose retained by the liverless animal were oxidized as glucose, or exerted the equivalent sparing action on metabolism of glucose a definite alteration in

the curve of the decreasing blood glucose should be obtained. Injection of comparable amounts of glucose produces definite alteration in the blood glucose curve following hepatectomy. We have obtained only questionable changes following administration of galactose. The amount of galactose that is apparently utilized after hepatectomy does not quantitatatively alter the metabolism of glucose. The foregoing considerations also indicate that the retained galactose is not quantitatively converted to glucose, since such conversion should alter the blood glucose curve, as is the case when fructose is administered. That galactose may form glycogen in the muscles without the intervention of the liver is a possibility that we are unable to rule out by our experiments. The amount of glycogen formed in the muscles from the galactose retained would be too small to be recognized as definite in this type of experiment. Administration of equivalent amounts of glucose would not be recognizable in the changes found in the glycogen content of the muscle. Any change in the glycogen content of the muscle of the hepatectomized dog would not be reflected in the glucose content of the blood.

Since essentially normal amounts of galactose were excreted by animals of which the liver was the site of extensive cirrhosis (with no acute degenerative changes in the liver) and by animals from which 50 to 70 per cent of the liver had been removed, it is obvious that no direct relationship exists between the amount of liver present and the utilization of galactose. These findings are quite in keeping with similar observations on other functions of the liver such as the regulation of the glucose content of the blood, deaminization and formation of urea, excretion of bile, and so forth. Within rather wide physiologic limits a small portion of normal liver is capable of maintaining all of the known functions of the entire organ with an efficiency that appears to be well within the observed normal variations. Degenerative changes of the hepatic cells, however, may greatly alter this picture, so that mild changes which cannot be detected by functional tests of the entire organ may cause complete cessation of certain functions when only a small portion of the liver is present.

The presence of acute changes in the liver, such as are produced by the administration of carbon-tetrachloride, chloroform, phosphorus or toluylenediamine definitely impairs the utilization of galactose. More galactose was excreted in the urine of these animals in the presence of such changes than when the same amount of galactose was injected before injury to the liver or after the animals had recovered from the effects of the hepatic poison. At the time of decreased retention of galactose, definite histologic evidence of degenerative changes was present in the liver and other functions were also impaired. The animals retained bilirubin in the blood, and the elimination of bromsulphalein was impaired. The amount of galactose excreted was roughly proportional to the extent of the hepatic injury but did not approach that returned by the hepatectomized dog unless

the animal was in the premortal stage from extensive hepatic destruction. In this respect utilization of galactose differs from utilization of glucose in the presence of necrosis of the liver. There is no gradual change in the maximal tolerance of glucose. Not until lethal injury to the liver has been produced does the maximal tolerance of approximately 2 grams of glucose for each kilogram of body weight per hour of the normal dog fall abruptly to the 0.75 gram tolerance of the hepatectomized dog.

SUMMARY

Galactose injected intravenously into normal dogs (500 mgm. for each kilogram of body weight) disappears from the blood in about two hours, and 10 to 30 per cent of the amount given appears in the urine. In the absence of the liver similar injections are followed by similar disappearance of galactose from the blood, but 50 to 60 per cent is recovered in the urine. There is greater delay in the clearance of galactose from the blood of nephrectomized animals, and still greater delay when the liver is also removed. Utilization of galactose is definitely impaired by hepatectomy but appreciable amounts appear to be utilized in the absence of the liver. This galactose is probably not converted to glucose, for it is without effect on the hypoglycemia of hepatectomized animals, and there is little sparing action on the blood glucose.

Removal of 50 to 70 per cent of the liver is without effect on the amount of galactose excreted in the urine following intravenous administration. The presence of acute degenerative lesions of the liver, such as are produced by carbon-tetrachloride, chloroform, phosphorus or toluylenediamine increases the amount of galactose recovered in the urine proportionally to the histologic changes present in the liver. Other physiologic evidence of impairment of hepatic function was present in the animals that gave evidence of decreased tolerance for galactose.

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ON THE ABSORPTION AND UTILIZATION OF CAROTENE AND VITAMIN A IN CHOLEDOCHOCOLONOSTOMIZED VITAMIN A DEFICIENT RATS[1]

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The present investigation is one of a series dealing with the rôle played by the bile in the transport of the fat soluble vitamins across the intestinal tract of the rat. Previous papers have dealt with vitamins A and D (1), (2). The present paper deals with the rôle played by bile in the absorption and utilization of carotene. The carotenes (α-, β-, and γ-) are now recognized as being precursors of vitamin A (3).

Certain evidence points to the fact that deoxycholic acid can form compounds with vitamin A and with the carotenes. Thus Shimizu and Hatakeyama (4) prepared a choleic acid compound of deoxycholic acid and vitamin A. The sodium, potassium and calcium salts were water soluble. Administration of these salts to vitamin A deficient mice led to the disappearance of the avitaminotic symptoms. von Euler and Klussmann (5) found, by fusing cholic or deoxycholic acid and carotene, and subsequently dissolving the melt in alkali, that the carotene was rendered water soluble and diffusible. Oleic acid favored the solubility of carotene to a lesser extent. The observations of von Euler and Klussmann have been confirmed in the present experiments.

Experimental. Young female rats bred under conditions which insured fairly low vitamin A reserves were weaned at the age of 28 days (wt. 40 to 60 gm.), and placed upon a diet (6) consisting of casein 18 per cent, Osborne-Mendel salt mixture 4 per cent, yeast 10 per cent, and cornstarch 68 per cent. During the first three weeks' period the casein was not extracted in order that the symptoms resulting from depletion would be delayed until after sexual maturity was reached. After that the casein was extracted according to the method recommended by Sherman and Smith (7). One-fifth of the yeast was irradiated in order to insure adequate amounts of vitamin D. Following the operation all animals were kept at constant temperature in individual metabolism cages. From the

[1] We are indebted to E. R. Squibb and Sons for the fellowship which made this experimental work possible.

[2] E. R. Squibb and Sons Fellow, 1934–35.

time of the opening of the vaginal orifice (40 to 90th day, average 60 days) daily vaginal smear determinations according to the method of Evans (8) were carried out. This test is now generally accepted as a criterion of vitamin A depletion (9). It was of particular value in the present work since it is one of the earliest signs of vitamin A depletion and its value as an index of avitaminosis A is not influenced by the present operative procedure.

There has been considerable divergence in the reported dosage of β-carotene which is necessary to restore the vaginal smear picture of vitamin A depleted rats to normal (9) (10). This is probably due to the differences in the experimental methods. The basal diet as well as the solvent used in administering the carotene are important variables. In the present experiment a daily intake of 10 γ of carotene was found to be sufficient to restore the vaginal smear picture to normal. In examining the vaginal smears it was noted often that after 2 to 3 weeks of straight cornified cells a more marked impairment of the vaginal wall manifested itself along the lines reported by Baumann and Steenbock (9). The cells became more necrotic. There was an accumulation of débris and later colloidal material, as well as, in some cases, a re-occurrence of leucocytes. Both this picture and the one in which only cornified cells were present were accepted as diagnostic of vitamin A depletion. The condition was easily differentiated from normal dioestrus. The occurrence of a vitamin A deficient vaginal smear picture for a period of 10 consecutive days was accepted as diagnostic of vitamin A depletion. Only those rats which met these requirements were used in the present experiments.

Internal bile fistulas were made by an anastomosis between the bile duct and the upper part of the descending colon essentially in accordance with the procedure which has been described previously (11), except that a small silver cannula was inserted into the bile duct and this in turn was sewed into the colon. By the use of this modified technique, about 85 per cent of the operations on a series of over 200 vitamin A deficient female rats were successful, while, in the previous work, a considerable number of the animals were jaundiced for varying periods of time as a result of the operation. By cannulating the bile duct at a level near the liver, and tying it directly below the cannula, the remaining part of the bile duct serves to transport the major part of the pancreatic juice into the duodenum. The silver cannula conducts the bile together with any pancreatic juice which may enter the bile duct at a high level into the lower colon. Some of the operated animals which received halibut liver oil to check the avitaminotic conditions lived as long as 3 to 4 weeks after the operation. They died from a hemorrhagic condition in the lower small intestines. Histological sections which were made from the livers of representative animals were normal. In another series of internal bile

fistula operations carried out on adult rats, the details of which will be published later, about 20 per cent of the animals showed marked ulceration with hemorrhage in the lower small intestines, while some of the animals lived in apparent health for periods as long as 10 months after the operation and did not show intestinal hemorrhage at autopsy.

The carotene used was prepared from carrot root.[3] It consisted of a mixture of the α- and β-forms (approximately 15 per cent α- and 85 per cent β-carotene). It melted at 173°. A 0.045 per cent solution was prepared by dissolving the required amount of carotene in petroleum ether and in turn adding this solution to either ethyl laurate or cottonseed oil. The petroleum ether was evaporated in vacuo. No appreciable decrease in the activity of carotene dissolved in these solvents takes place under the conditions used in these experiments. The carotenes are stable in these solvents (12). The solutions were kept in an atmosphere of nitrogen in the ice chest. A fresh solution was prepared weekly. Parallel experiments on control animals were carried out to test the potency of the carotene solutions. The carotene was administered daily to the experimental animals beginning 24 hours after the operation. The orally treated groups received the carotene solutions by stomach tube.

The deoxycholic acid-carotene mixture was prepared as follows: the required amount of a petroleum ether solution of carotene was added to an aqueous solution of deoxycholic acid which had been neutralized to pH 8.0 by means of sodium hydroxide. The petroleum ether was removed by heating to 80° in the absence of oxygen. This will be designated as preparation no. 1. The second carotene-deoxycholic acid solution was prepared as follows: one equivalent of carotene and 2 equivalents of deoxycholic acid were fused at 175° in the absence of oxygen. The cooled melt was dissolved in dilute sodium hydroxide solution and the mixture was passed through thin parlodion sacks in accordance with the procedure used by Greenberg and Gunther (13). The glycodeoxycholic acid-carotene solution was prepared by adding equivalent amounts of glycine and deoxycholic acid to a dilute solution of sodium carbonate. The mixture was heated to 60° to effect solution. To this was added a solution of carotene dissolved in petroleum ether or cottonseed oil, the mixture was shaken and heated to 80°. The petroleum ether was removed in vacuo. This was designated as preparation no. 3. The fourth preparation was made by fusing carotene and deoxycholic acid. The melt was dissolved in sodium hydroxide solution, then neutralized to pH 8.0 and ultrafiltered. The solutions were kept under an atmosphere of nitrogen in an ice chest. Fresh solutions were prepared each 48 hours during the course of the experiments.

[3] The carotene was kindly supplied by Dr. Gordon McKinney.

The experimental data are summarized in table 1. The unoperated animals in groups I to V inclusive served as controls to establish the potency of the carotene preparations as well as to indicate the rate of response of the vaginal smear picture. The rats in groups VI to XIII inclusive were operated in the manner described and were given either carotene or halibut liver oil concentrate in the manner and dosage indicated in table 1.

It will be noted from the data given in table 1 that the majority of the animals of groups I to V showed a positive response to the carotene prior to the fifth day (about 50 per cent of groups I to IV responded prior to the fourth day of treatment), while practically all responded prior to the seventh day. On the other hand, none of the group VI rats showed a response, and only two, or 6 per cent, of group VII responded to the orally administered carotene dose. The two which responded died shortly after a positive vaginal smear picture was exhibited. Of the animals in group VI, 70 per cent lived more than three days, while 50 per cent lived 5 or more days after the operation. In group VII nearly 60 per cent lived 4 or more days after the operation, while 33 per cent lived 5 or more days. Two lived 7 days after the operation. In general, the results, when contrasted with the control groups, indicate that little or no absorption of carotene took place from the intestinal tracts of the operated rats. If absorption did take place, the amount was less than 10 γ per day, the minimum dose which was found necessary, in these experiments, to change the vaginal smear picture to normal. The results indicate, moreover, that the presence of bile in the lower colons of the operated rats does not lead to absorption of carotene from this region of the intestinal tract.

Administration of daily doses of 45 γ of carotene dissolved in 0.3 cc. of oil subcutaneously led only 1 animal out of 14 (group VIII) to respond, while, when 90 γ of carotene dissolved in 0.2 cc. of oil were similarly administered (group IX), 17, or 77 per cent, responded. These experiments indicate two things: *a*, absorption of carotene administered subcutaneously in cottonseed oil is markedly retarded when dissolved in low concentration. When the concentration in this solvent is increased sufficiently, some absorption takes place; *b*, the results are in contrast to those obtained on the members of groups VI and VII to which the carotene was given orally. The carotene when absorbed is active in relieving the avitaminotic condition; the negative data indicate failure of absorption either from lack of bile when the carotene is administered orally or from too much fat as indicated in the experiments in which carotene was administered subcutaneously in low concentration. Additional experiments which were carried out on adult external bile fistula rats, the details of which will be published later, indicate that the bile does not serve as a channel of excretion of vitamin A or carotene in appreciable amounts in the dosages administered in these experiments. Failure of the animals in group VIII to re-

TABLE 1

	CONTROL SERIES. VITAMIN A DEFICIENT FEMALE RATS. NOT OPERATED					EXPERIMENTAL SERIES. VITAMIN A DEFICIENT FEMALE RATS. OPERATED (CHOLEDOCHOCOLONOSTOMIZED)								
	Carotene given orally daily		Carotene given subcutaneously daily		Carotene given intraperitoneally daily	Carotene given orally daily		Carotene given subcutaneously daily		Carotene given intravenously	Carotene bile salt mixture given orally daily		Halibut liver oil concentrate given orally daily	No treatment
Group number	I	II	III	IV	V	VI	VII	VIII	IX	X	XI		XII	XIII
Total number of animals in group	12	20	13	37	6	12	33	14	22	8	25		7	9
Dosage of carotene (γ)	45	90	45	90	90	45	90	45	90	500–800 suspended in glucose solution			5000 vit. A rat units	None
Oil (cottonseed oil) or ethyl laurate administered, cc.	0.1	0.2	0.1	0.2	0.2	0.1	0.2	0.3	0.2		0.2		0.1	0.2
Animals reacted positively														
Day of treatment:														
1st day	1	0	0	0	0	0	0	0	0	0	0		0	0
2nd day	2	4	4	7	1	0	1	0	5	3	1‡	2**	4	0
3rd day	4	6	4	15	1	0	1	1	6	0	2‡	4*	0	0
4th day	3	6	3	10	0	0	0	0	2	2	2**	3¶	0	0
5th day	2	4	1	4	3	0	0	0	2	0	0		0	0
6th day	0	0	0	1	1	0	0	0	0	0	0		0	0
7th day	0	0	0	0	0	0	0	0	0	0	0		0	0
8th day	0	0	0	0	0	0	0	0	2	0	0		0	0
Total number of animals reacted	12	20	12	37	6	0	2	1	17	5	14		4	0
Animals did not react. Died														
Day of treatment:														
1st day	0	0	1	0	0	0	0	3	0	2	0		1	2
2nd day	0	0	0	0	0	4	12	1	3	0	1‡	5**	1	3
3rd day	0	0	0	0	0	2	10	4	1	1	2‡	2**	1	1
4th day	0	0	0	0	0	3	4	4	0	0	1*		0	2
5th day	0	0	0	0	0	2	3	1	1	0	0		0	1
6th day	0	0	0	0	0	1	2	0	0	0	0		0	0
7th day	0	0	0	0	0	0	0	0	0	0	0		0	0
Total number of animals not reacting	0	0	1	0	0	12	31	13	5	3	11		3	9
Animals which reacted, per cent	100	100	92	100	100	0	6	7	77	63	56		57	0

* Preparation no. 1 was administered.
** Preparation no. 2 was administered.
‡ Preparation no. 3 was administered.
¶ Preparation no. 4 was administered.
(See text for details relating to these preparations.)

spond was not due to the lower dosage (45 γ) since response to this dosage was shown by the rats in the two control series. When 500 to 800 γ of carotene as a single dose suspended in glucose were administered intravenously to the animals in group X, 63 per cent showed a positive response.

Oral administration of the several carotene-deoxycholic acid compounds was followed by a positive response in 56 per cent of the animals in group XI and essentially the same percentage of the operated rats in group XII responded to oral administration of halibut liver oil concentrate although no bile acids were administered to these animals. Of 5 animals which received the carotene-deoxycholic acid preparation no. 1, 4 reacted positively on the third day, while the fifth died on the same day. Four of the rats which received preparation no. 2 showed a positive response before the fifth day, while the seven which did not respond died prior to the fourth day of the experiment. Half of the six animals to which preparation no. 3 was administered exhibited a positive response prior to the fourth day, while those which did not respond died prior to the same time. All of the animals to which preparation no. 4 was fed reacted positively on the fourth day. These experiments not only confirm previous observations from this laboratory (1), which showed that vitamin A can be absorbed from the intestinal tracts of rats from which bile is excluded by ligation of the common bile duct, but also indicate that deoxycholic acid is an active agent in the transport of carotene across the intestinal tracts of choledochocolonostomized rats when fed orally. The rats in group XIII served as controls, indicating that when neither carotene nor vitamin A was administered, the vaginal smear response continued negative. The data obtained in the animals in this group show that the operation itself does not affect the vaginal smear picture.

A few general remarks on the preceding discussion are pertinent. Although the rats were kept in separate metabolism cages in order to minimize the possibility of obtaining bile orally from contact with excreta, it was not possible to eliminate this factor entirely. Attempts were made to fit collars around the necks of a number of rats in such a manner that it would be impossible for them to lick their paws. In another series the animals were suspended in a jacket from the top of the cage so that the front feet could not touch the screen bottom of the cage. However, these procedures proved objectionable and led to an earlier death of the animals. In no case in which this added precaution was taken was a positive response obtained when carotene alone was administered orally to the bile fistula rats. It is also possible that small amounts of bile remained in the intestinal tracts of the operated animals during the 24 hour period following the operation, and this factor may, in some cases, have been responsible for the absorption of small amounts of carotene. Due to the high mortality resulting from the avitaminosis, it was not practicable to postpone

the time when the feeding of carotene was begun. However, the amount of bile remaining in the intestinal tract was apparently small since most of the animals when fed carotene orally did not respond.

A number of workers have shown that the rat can absorb considerable amounts of carotene when this substance is orally administered (14). Moore (15) fed two rats a daily dose of 8,300 γ of carotene per day and found 90 per cent, or about 7,500 γ, to be utilized, indicating that the normal rat's ability to absorb carotene is very great. The dosage given in the present experiments was very low when contrasted with this amount but is still 5 to 10 times the necessary dose. The extreme contrast between the two results indicates marked impairment in the intestinal absorption of carotene in the bile fistula animal. In the absence of bile from the intestinal tract, these small doses were not absorbed to the extent that they were therapeutically effective.

The work of Ahmad (16) indicates that the absorption of carotene from the intestinal tract is considerably affected by the fat content of the diet. Rats which were fed a low fat diet excreted about 90 per cent of the carotene which was administered in ethyl laurate solution when 500 γ were fed, and about 40 per cent when 100 γ were fed in palm oil solution. The absorption was practically complete when the basal diet contained 10 per cent of fat.

The following series of experiments were carried out on normal vitamin A depleted rats in order to determine whether the fat content of the diet plays any important rôle in the absorption of carotene when low levels of carotene are administered orally. The animals in all groups were maintained on the same basal diet (fat content of about 3 per cent) as previously described. In addition, they received carotene-deoxycholic acid solutions or fat in the amounts as given below. Unless otherwise stated, the carotene was dissolved in cottonseed oil in such a concentration that 0.1 cc. of oil represented the daily dose of carotene which was administered. This was fed orally unless otherwise stated. The protocols are: Group XIV—15 γ carotene. Group XV—same as the above except that the basal diet contained 20 per cent lard. Group XVI—same as group XIV except that deoxycholic acid was also fed. Group XVII—5 γ carotene. Group XVIII—same as group XVII except that the basal diet contained 20 per cent lard. Group XIX—same as group XVII except that deoxycholic acid was fed along with the carotene. Group XX—5 γ carotene dissolved in ethyl laurate. Group XXI—same as group XX except that the basal diet contained 20 per cent lard. Group XXII—same as group XX except that deoxycholic acid was fed along with the carotene. Group XXIII—5 γ carotene administered subcutaneously.

The deoxycholic acid solutions were prepared by saturating a 1 per cent sodium carbonate solution with deoxycholic acid and shaking an equal

volume of this solution with the oil solution containing the carotene. The emulsion was administered by stomach tube in a dosage of 0.2 cc. This represents 20 mgm. of deoxycholic acid. The animals were weighed twice weekly.

In all cases the growth response, as shown in figure 1, was slightly lower in the groups of animals which were maintained on the fat-containing diet

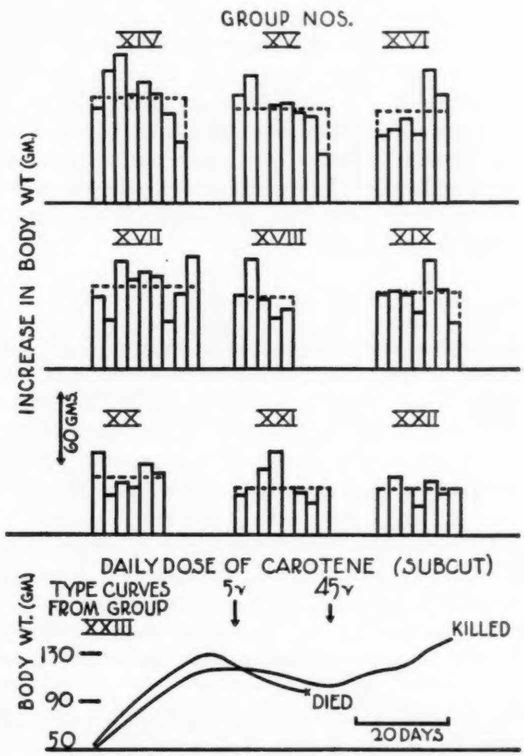

Fig. 1

than in the groups which received the basal ration. This was likewise true of the animals which received deoxycholic acid. However, the differences are not significant. The important point is that the growth increments in the various groups were not significantly different indicating that under the conditions of these experiments the fat content of the diet did not influence materially the absorption of carotene. Balance experiments which were carried out on representative members of the above groups

showed that the carotene which was excreted in the feces constituted less than 10 per cent of the total fed. Deoxycholic acid is one of the most toxic of the bile acids. In order to rule out the factor of toxicity in groups XVI, XIX and XXII, complete autopsies were performed. In no case were pathological lesions found that could be attributed to this factor.

The data obtained on the animals in groups VIII and IX indicate that the concentration of carotene when administered subcutaneously in cottonseed oil may be an important factor in determining its absorption. To test this point further, the experiments on group XXIII were carried out (see fig. 1). When 5 γ of carotene dissolved in 0.1 cc. of cottonseed oil were administered subcutaneously daily for a period of 16 days, no response indicative of carotene utilization was obtained. Seven of the nine rats had died by the sixteenth day. The dosage was then increased to 45 γ dissolved in the same amount of cottonseed oil. One animal died shortly afterward, but the other showed an immediate gain in weight and continued so for the remainder of the experiment.

SUMMARY

1. Using the vaginal smear technique as a criterion for the absorption of carotene and of vitamin A, a series of experiments was carried out on choledochocolonostomized and unoperated vitamin A deficient rats.

2. The unoperated animals responded to the carotene therapy when administered orally or subcutaneously within 6 days. About one-half of the members of these groups responded within 4 days after treatment was begun.

3. The choledochocolonostomized rats did not respond when carotene was administered orally, but response was obtained when this substance was administered subcutaneously in certain concentrations.

4. The operated animals showed a positive response to carotene when this substance was administered along with glycodeoxycholic or deoxycholic acid.

5. The experiments indicate that these bile acids can function as carriers of carotene across the intestinal tract of the rat.

6. The fat content of the diet, within the limits fed, does not materially influence the absorption of carotene when the latter substance is fed orally at a daily level of 5 to 15 γ.

7. In confirmation of previous work on icteric rats, it is shown that vitamin A when fed orally is absorbed in sufficient amounts by the internal bile fistula rat to correct the vaginal smear picture.

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THE UTILIZATION OF CAROTENE BY JAUNDICED AND PHOSPHORUS TREATED VITAMIN A DEFICIENT RATS[1]

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Previous work from this laboratory has indicated that the administration of viosterol to jaundiced rachitic rats does not aid calcification as it does in the normal rachitic rat (1). These findings were interpreted as indicating probable injury to the osteogenic cells. On the other hand, administration of vitamin A to icteric rats restores the vaginal smear picture to normal (2). There is abundant evidence that extensive damage is caused to various tissues of the body, especially the liver, as a result of jaundice (3). It seemed of interest to determine whether the conversion of carotene which takes place in the normal rat is inhibited in the icteric rat. The available evidence points to the liver as the locus for the transformation of carotene to vitamin A (4), although direct proof is still lacking. The livers of carotene fed rats show an increased antimony chloride reaction over depleted control rats (5), and the spectroscopic analysis of the non-saponifiable fraction of the livers of carotene fed animals shows absorption bands which are characteristic of vitamin A (6). The great bulk of the vitamin A stores of the body is found in the liver. It would appear probable that injury to the liver may interfere with the conversion of carotene to vitamin A and possibly also with the storage of the latter substance.

Experimental. The experiments were carried out along the lines which have been indicated in the previous paper (7). The condition of icterus was induced by doubly ligating and sectioning the common bile duct at a level near the liver. Vitamin A deficient female rats were employed, and the vaginal smear picture was used as an index of carotene utilization. The carotene was administered in an ethyl laurate or cottonseed oil solution with the exception of that which was administered intravenously, in which case it was injected in the form of a colloidal suspension in 5 per cent glucose solution. Each rat received 0.2 cc. of a 0.045 per cent solution of carotene (90 γ) per day. Some of the animals in the

[1] We are indebted to E. R. Squibb and Sons for the fellowship which made this work possible.

[2] E. R. Squibb and Sons Fellow, 1934-35.

unoperated control groups, I and II, received 45 γ of carotene per day. The designations of the other groups as well as the mode of administration of carotene and of halibut liver oil are given in table 1.

TABLE 1

	CONTROL SERIES. NOT OPERATED			JAUNDICED ANIMALS					
	Carotene administered subcutaneously	Carotene administered orally	Carotene administered intravenously	Carotene administered orally	Carotene administered subcutaneously	Carotene administered intraperitoneally	Carotene administered intravenously	Halibut liver oil administered subcutaneously	Halibut liver oil administered orally
Group	I	II	III	IV	V	VI	VII	VIII	IX
Number of animals in group	50	32	4	23	32	12	5	9	10
Animals reacted positively									
Day of treatment:									
1st day	0	1	0	0	0	0	0	0	0
2nd day	11	6	0	0	0	0	0	1	3
3rd day	19	10	1	1	0	0	0	2	1
4th day	13	9	2	0	0	0	0	1	2
5th day	5	6	0	0	0	0	0	0	0
6th day	1	0	0	0	0	0	0	0	0
7th day	0	0	1	0	0	0	0	0	0
Total	49	32	4	1	0	0	0	4	6
Animals did not react. Died									
Day of treatment:									
1st day	1	0	0	0	0	0	0	0	0
2nd day	0	0	0	9	11	2	2	0	1
3rd day	0	0	0	5	12	2	0	2	0
4th day	0	0	0	2	1*	4	1	1	2
5th day	0	0	0	1	2,* 1	2	0	2	1
6th day	0	0	0	1	1,* 3	2	0	0	0
7th day	0	0	0	3	0	0	1	0	0
8th day	0	0	0	1	1	0	1	0	0
Total	1	0	0	22	32	12	5	5	4

* The starred numbers indicate that a dosage larger than that indicated in the text was administered to the number of animals given.

As seen from the data given in table 1, of 50 animals (group I) to which carotene was administered subcutaneously, 49 reacted positively; all of the 32 rats in group II, which received carotene orally, responded simi-

larly; the 4 animals in group III, which received carotene intravenously, did likewise. On the other hand, of the jaundiced series, one of 23 rats (group IV) responded to the carotene when the substance was administered orally, while none of the animals in the groups to which carotene was given subcutaneously (group V), intraperitoneally (group VI), or intravenously (group VII) showed a response in the vaginal smear picture. These rats lived for comparatively short periods of time. Of 9 rats to which halibut liver oil was administered subcutaneously (group VIII), 4 exhibited a positive response, and 6 of the 10 animals in group IX, which received halibut liver oil orally, responded.

These experiments not only established the potency of the carotene, but also confirmed previous work (2) that vitamin A is absorbed from the intestines of the jaundiced vitamin A deficient rat when orally administered, and is active in restoring the vaginal smear picture to normal. The failure of nearly all of the icteric vitamin A deficient rats to respond to the administration of carotene is indicative of the inability of these animals to transform carotene to vitamin A.

Additional information on this point was obtained by inducing liver damage by means of phosphorus. Slight to moderate liver damage resulted when 0.1 to 3.0 mgm. of phosphorus, dissolved in olive oil (1 cc. = 10 mgm. P), were administered to vitamin A deficient rats for periods of 6 to 7 days. Extension of the treatment beyond this time, or increased dosage, led to increased mortality in the animals. Subcutaneous administration of phosphorus in the doses indicated in table 2 does not alter the vaginal smear picture of vitamin A deficient rats nor does it interfere with the usefulness of this test as a criterion for carotene absorption.

The total number of animals employed in these experiments is comparatively small and the results must therefore be interpreted with some caution. The data given in table 2 show that of 5 rats which received 2 mgm. or less of phosphorus daily, 4 responded to the carotene, while 3 of 5 animals which received a daily dosage of 3 mgm. of phosphorus responded to the carotene treatment. The data are interpreted as indicating that the transformation of carotene to vitamin A is apparently somewhat inhibited as a result of the phosphorus treatment. Due to the extreme toxicity of the phosphorus, together with the advancing avitaminotic condition of the animals, it was not found practicable to extend the period of treatment or to increase the phosphorus dosage.

No demonstrable interference with the conversion of carotene to vitamin A resulted when vitamin A deficient rats were treated with chloroform, benzene or carbon tetrachloride in the dosage which it was found possible to administer. With the exception of the benzene treated animals, liver damage resulted from these treatments. Apparently the damage is not sufficient, however, to seriously interfere with the function of the liver in

converting carotene to vitamin A. These experiments recall those of Greenberg (8) who found that the action of parathormone was decreased markedly in dogs poisoned by phosphorus but not when the animals were treated with the other above mentioned poisons.

TABLE 2

Phosphorus treated series

RAT NUMBER	DOSAGE OF PHOSPHORUS	PHOSPHORUS TREATMENT	CAROTENE GIVEN	BODY WEIGHTS		RESULTS
				Initial	Final	
	mgm.	*days*	*days*	*gm.*	*gm.*	
250	2.0	1 to 5 and 10	None	123	99	Straight cornified smear picture throughout
307	3.0	1 to 3	None	66	68	Straight cornified smear picture throughout
263	3.0	1 to 4	None	100	103	Straight cornified smear picture throughout
306	3.0	1 to 2	None	62	64	Straight cornified smear picture throughout
238	1.0	1 to 3	1 and 2			Vaginal smear picture corrected 3rd day. Xerophthalmia present
191	2.0	1 to 4	2 to 4			Vaginal smear picture corrected 3rd day. Lived 5 days
239	2.0	1 to 3	2			Vaginal smear failed to respond
260	2.0	1 to 4	3 and 4	70	75	Vaginal smear picture corrected 5th day
243	2.0	1 to 7	2 to 6	118	116	Vaginal smear picture corrected 3rd day
273	3.0	1 to 3	2 and 3	91	89	Vaginal smear picture corrected 3rd day
252	3.0	1 to 5	4	108	106	Vaginal smear picture corrected 3rd day
294	3.0	1 to 4	2 to 4	104	105	Vaginal smear picture corrected 3rd day
303	3.0	1 to 4	2 and 3	87	85	Vaginal smear failed to respond. Lived 5 days
297	3.0	1 to 4	2 to 4	84	86	Vaginal smear failed to respond. Lived 6 days

SUMMARY

1. Using the vaginal smear picture response in rats as a criterion for the conversion of carotene to vitamin A, it is shown that, irrespective of the channel used in administering the carotene, little or no conversion takes place in the vitamin A deficient icteric rat.

2. Treatment of vitamin A deficient rats with phosphorus apparently

leads to a decreased ability to transform carotene into vitamin A. No such effect was obtained when the rats were treated with benzene, chloroform or carbon tetrachloride.

3. While the evidence is not directly conclusive, the experiments point to the possibility that the liver is the organ in which carotene is chiefly converted to vitamin A.

4. Previously reported experiments have shown that vitamin A, when administered orally to vitamin A deficient icteric rats, is absorbed. The conclusions, viz., that vitamin A can be absorbed in the absence of bile from the intestinal tract, have been confirmed on icteric rats.

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PROLONGATION OF PREGNANCY IN THE RAT BY THE INJECTION OF HUMAN PREGNANCY URINE EXTRACT

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The part played by the corpus luteum in parturition in the rabbit has been recently studied by Snyder (1934), who induced ovulation and the consequent production of a fresh crop of corpora lutea by the injection, near term, of human pregnancy urine extract. Normal parturition did not occur and, in most cases, the fetuses were retained throughout the life-span of the new corpora lutea, 15 days.

The luteinization of the rat's ovaries has been stimulated by different methods and the effect on the course of gestation observed by a number of investigators. The injection of extracts of anterior lobe (Teel, 1926; Evans and Simpson, 1929; Hain, 1933) or the transplants of anterior lobe (Teel, 1926; Engle and Mermod, 1928; Evans, 1928; Hain, 1933) interrupted pregnancy or caused its prolongation, depending on the dosage and the time of treatment. The removal of the anterior lobe of the rat prolongs the life of the corpora lutea already present and, taking advantage of this fact, Pencharz and Long (1933) performed the operation on rats between the 11th and 20th days of gestation. As a result the young were retained three to four days beyond term. Nelson et al. (1930) delayed parturition for from 48 to 150 hours by injecting different extracts of corpus luteum on the 17th to the 18th days. The injection of human pregnancy urine used by Levin et al. (1931) and Hain (1933) caused the prolongation of pregnancy, presumably due to the stimulation of the production of a new set of corpora lutea. Hain (1934) suggests that some other factor may be responsible. In general, these workers agree that it is the presence of the secretion of the corpora lutea, whether given as an extract or produced by the rat's own ovaries, which is responsible for the prolongation of the gestation period and, as a result, abnormally large fetuses.

Evans and Long (1923) refer to the histological changes which are very evident in the corpora lutea of the rat's ovary 12 hours before parturition and Parkes (1929) states that in the mouse the corpora cease to function 2 days before term. On the basis of these observations as well as of Snyder's (1934) work on the rabbit, it appeared that one injection of human preg-

nancy urine extract could be so timed that the function of the induced corpora lutea would synchronize with the retrogression of those of pregnancy. Hoopes (1934) reported on the standardization of a method by which one injection of 75 rat units of Antuitrin-S, the Parke, Davis Company's preparation, when given on the 19th day of gestation, regularly extended its duration and postmature fetuses were demonstrated.

The present paper reports these results with Antuitrin-S in more detail, gives additional experiments and also describes the condition of the ovaries as revealed by histological study.

METHOD. Vaginal smears were made each morning of rats in the mating cages and the day that sperms were found was reckoned as day one of gestation. The placental sign was usually observed on the 13th or 14th day but occasionally it was seen on the 12th or the 15th. An error of 2 or even 3 days might be made if the sign alone were used for dating the pregnancy. In our colony, parturition occurred most frequently on the 22nd or the 23rd day. Consequently pregnancy was regarded as prolonged if it continued beyond 23 days and if the fetuses were postmature.

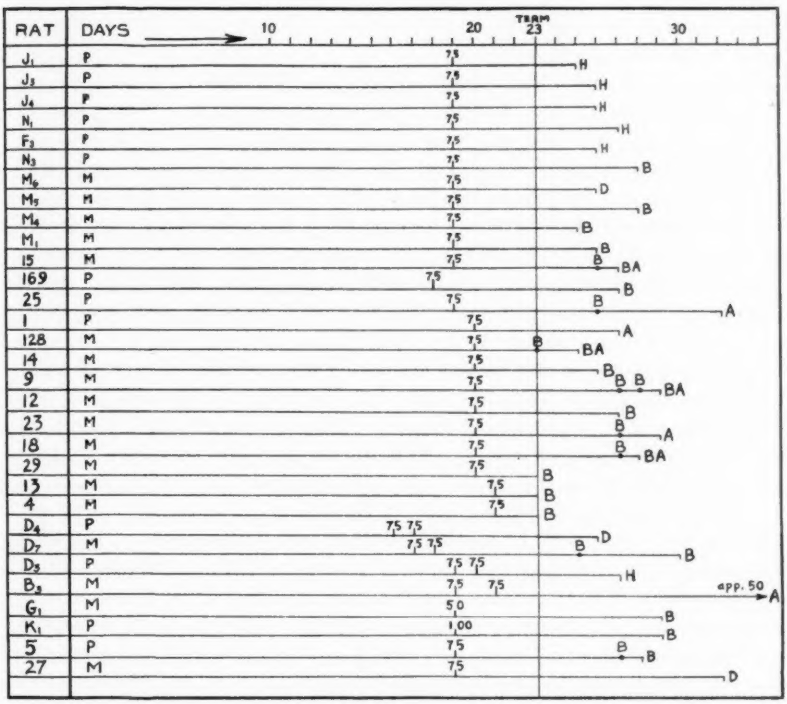
Although there were no fatalities, it was desired not to give an unnecessarily large amount of extract or to give it more frequently than was necessary. Tests showed that 75 rat units given on the 19th day or even on the 20th were effective in delaying parturition but when given on the 21st day a normal litter was born at term. The product used was standardized so that 1 cc. contained 100 rat units. This amount cannot well be compared with that given by Levin et al., who gave 1500 to 1700 mouse units of their extract in 2 or 3 doses between the 14th and 21st days. Hain injected an extract, as well as unconcentrated human pregnancy urine, on 4 or 5 successive days selecting different periods during gestation.

RESULTS AND DISCUSSION. Significant data in regard to the entire series of 31 animals are presented in the table. Special interest attaches, however, to 14 animals injected with 75 rat units on the 19th day, to 8 injected on the 20th day and to 1 injected, by mistake, on the 18th instead of the 19th day. All but one of these 23 pregnancies continued, when not purposely interrupted, for from 3 to 9 days past term. Nineteen of these rats attained their maximum weights on the 22nd or 23rd days. This weight was occasionally held for a day or two and then fell at first slowly and then rapidly, as parturition began. Early loss might well be due to the fact that they ate very little after the 23rd day but stayed crouched in one corner of the cage, as though asleep. Vaginal bleeding began on the day that the maximal weight was reached, or 1 or 2 days after, in 17 of the 23 cases; in one there was no bleeding and in the others it appeared later, even on the 27th day. In rat 29, which was an exception in that she cast a normal litter at term, a few red cells were seen microscopically the day before parturition. The gross bleeding was characteristic of retention of the

fetuses and it undoubtedly came from the placentas. As parturition is further delayed, the vaginal discharge becomes dark and mucoidal, due to the autolysis of dead fetuses. In uninterrupted cases, the casting of the litter began on the 25th day in 2 rats, on the 26th in 7 and on the 27th

TABLE 1

Summary of results following injection of Antuitrin-S into 31 pregnant rats



Injections are indicated by short vertical lines above horizontal; dosage above in R.U. The following symbols are used: P—primipara. M—multipara. H—hysterotomy, fetuses in utero. B—birth of one or more fetuses. D—death of mother, fetuses in utero. A—autopsy, fetuses in utero. Spermatozoa were seen in all rats except N-1, M-1, and B-3; the placental sign in all except J-1, J-4, N-3, M-6, 15, 169, 14, and B-3.

in 4. There was one exception in rat 128, which expelled one dead fetus on the 23rd day. Parturition was always protracted and continued from 2 to 6 days; in one instance, an hour and forty-five minutes was required to expel one fetus. Difficulty was experienced in rescuing the young as a

mother often began eating one before it was entirely out of the vagina. Undoubtedly, a number were not seen, but an appreciable drop in weight, over night for example, indicated that they had been born. The mother would become lively and eat well after most of her litter had been expelled, though at autopsy there might still be a dead fetus in utero. A significant relationship was not noted between the prolongation of pregnancy and the parity of the animals, as 16 of the 28 prolongations were in primiparae and 12 in multiparae. Snyder (1934) had more abortions in the primiparous rabbits injected with Antuitrin-S and a higher per cent of prolongations in the multiparous.

Condition and size of the fetuses. In only one instance was a living fetus born on the 25th day (to M-4) and it died soon after birth. Living fetuses of normal size were removed from J-1 at operation on the 25th day and also 8 living, postmature ones from J-3. The average crown-rump length of the latter was 5.2 cm. in contrast to the normal 4.2 to 4.5 cm.; their average weight was 6.2 grams in contrast to the normal average of 5.5 grams. Greatly enlarged fetuses were never expelled but were found in the uterus usually with fetuses of normal or less than normal size. J-4 is especially interesting because in the litter of 4, all were large and postmature. Their average formalin length was 5.2 cm. and the average weight 8.2 grams. As previously stated (Hoopes, 1934), there were other evidences of postmaturity as shown by the vibrissae, the skin and the extremities. The young of an uninjected litter mate, averaged 4.5 cm. in length and 6.5 grams in weight. This weight, which is high for a control, is explained by the fact that the rats had already suckled when found, as well as the fact that they had been in formalin. In contrast to J-4, no. 128, injected with 75 R. U. on the 20th day, cast 1 dead fetus on the 23rd day and 3 more of normal size on the 24th and 25th days. She was killed on the evening of the 25th day and 3 fetuses with discolored amnions were in the uterus. Two were only slightly enlarged while the third measured 5.1 cm. and weighed 9.15 grams. This was the largest fetus of our series and was comparable in size and appearance to a normal rat 48 hours old. Repeatedly, the enlarged fetuses expelled by different rats appeared compressed, especially the head, and elongated as though they had been subjected to unusual pressure. This was particularly noticeable in rat 15. She expelled 6 fetuses on the 26th and 27th days, and a 7th was found in utero when she was autopsied on the 27th. The average weight of 4, not partially eaten, was 6.5 grams. At least, for 2 of them, it took 25 to 30 minutes to complete the expulsion, after a part of the fetus was out of the vagina.

Condition of the uterus. An appreciable quantity of darkened blood was sometimes found in the uterus at autopsy, indicating the separation of placentas. On the other hand, portions of the maternal side of placentas

of fetuses, born on the 27th day were fresh and vascular, as though recently detached.

Injury to the uterine wall was less common than Snyder (1934) found in the rabbit. Although abnormally distended by enlarged dead fetuses, usually the uterus readily contracted when they were removed. In 7 cases the wall of the uterus, examined at autopsy after parturition, was thin and contained hemorrhagic areas. A histological examination was made of the uterus of no. 25 which contained 4 degenerating fetuses at autopsy on the 32nd day, and of no. 169, autopsied also on the 32nd day, 5 days after expelling her last fetus. In each of these the uterus appeared uninjured. Two rats, D-7 and G-3, again became pregnant indicating a normal uterus. A peculiar condition was found in B-3, injected on the 19th and 21st days with 75 rat units of the extract. Judging from her maximum loss of weight, 9 grams, it is doubtful whether any young were born. She gradually gained, though there was continuous hemorrhage, until on the 49th day her weight was 41 grams more than on the 23rd day. She was autopsied on the 50th day. The uterus was greatly hypertrophied and appeared as 3 hard lumps and there were adhesions between it and the abdominal organs. The wall was extremely thin and the lumps contained closely impacted, degenerating fetuses.

Condition of the ovaries. The corpora lutea of the rat and mouse, in contrast to those of most mammals, remain as substantial, fairly well preserved structures so that 4 or 5 sets may be seen in the ovary of a non-pregnant animal. However, pregnancy not only inhibits ovulation but by the 20th day has caused the resolution of other corpora (Long and Evans, 1923).

It is a matter of much interest to determine how closely histological changes in the corpora lutea of pregnancy and in the induced corpora can be correlated with the physiological manifestations, delayed parturition and the development of postmature fetuses. With this purpose in view, the ovaries of experimental animals were carefully examined at autopsy. In addition, 1 was removed from an injected animal on days 20, 21, 22, 24, 25, 26, 27, 28 and 32, fixed in Bouin's solution and prepared for histological study. Serial sections of an entire ovary of 2 different rats killed on the 25th day and of the ovary of 1 killed on the 26th day, were studied. The 25th day is important, since living fetuses were never found later, and the 26th is especially significant because it is the day on which parturition most frequently started. As was previously stated, rats injected on the 21st day cast their litters on the 23rd and it is inferred that 2 days is not sufficient time for the induced corpora lutea to become functional. No. 29, the only animal injected on the 20th day in which parturition was not delayed, had 4 large follicles in each ovary and large old corpora. For some reason luteinization of the follicles had not occurred and a consequent prolongation of pregnancy. There was no indication of luteinization of

follicles in the ovaries of a rat injected on the 19th day and killed on the 20th nor could deterioration of the corpora lutea of pregnancy be discerned. There were, however, rather large follicles present.

The ovary of J-2, taken on the 21st day, showed many large follicles in the early stage of luteinization. They were so numerous that 8 were counted in one section. The cells, in a state of active division, formed a mass 5 to 6 deep about the wall of the follicles. Retained ova could be seen in some. The corpora lutea of pregnancy were conspicuous for their size and for the well defined capsules which easily delimited them from another set of corpora, belonging to a previous ovulation. Vacuolation of their cells had begun and there was some penetration of fibrous tissue. The walls of the luteinized follicles had increased appreciably in thickness by the 22nd day (D-7) and the difference between these and those of pregnancy was decidedly apparent. The lutein cells half filled the follicle by the 24th day (J-1). Those of pregnancy showed marked retrogression by their faint staining and vacuolation. The gland also contained much fibrous tissue. Serial sections were made of an ovary of J-3 taken on the 25th day, when postmature living fetuses were in utero. The induced corpora still had a central cavity but this was not significant, since some of those of pregnancy were not solid. They had the appearance of being actively secreting glands in contrast to the markedly retrogressing corpora of pregnancy. Some follicles showed a cystic, crescentic invasion of lutein cells, such as Engle and Smith describe and picture (1929). There were a number of large follicles containing ova. An ovary of N-1 was removed on the 26th day. Her drop in weight indicated that fetuses had been expelled on the 25th to the 26th day, but two dead ones were found in utero at autopsy. The induced corpora lutea were nearly solid and they showed, possibly, some slight signs of retrogression. In a few instances a degenerate ovum was imbedded in the gland. No. 15 cast 7 fetuses, 2 of them larger than normal, on the 26th and 27th days and a third was found in the uterus at autopsy on the 27th. Sections of the ovary, in addition to the retrogressing corpora of pregnancy and the induced ones, showed early luteinization of some follicles. Large follicles were also present but they were over-distended, the thecal cells had disappeared and only a thin layer of granulose cells could be seen. An ovary taken on the 28th day was not significantly different from the one just described. Rats 169 and 25 were each autopsied on the 32nd day: the first, 5 days after parturition was completed; the second after a part of the litter had been expelled but while 4 large degenerate fetuses were still in the uterus. There were 2 sets of corpora in the ovaries of each animal: those of pregnancy very degenerate, and the induced corpora, apparently normal.

The histological study of the ovaries of these typical cases, therefore, gives no conclusive evidence of the retrogression of the induced corpora

lutea even up to the 32nd day. If they are comparable to the corpora of pseudo-pregnancy, their function should cease by the 27th to the 35th day, dating from the day of the injection of the extract on the 19th day. In the rat, according to Long and Evans (1923), the corpora of pseudo-pregnancy function for from 8 to 16 days. It is possible that, when littering began on the 26th or 27th days, as was most frequently the case, these glands were no longer active or parturition may have been due to the detachment of the placentas, as indicated by the external bleeding. Long retention of the fetuses was associated with injury to the uterine muscle.

Occurrence of ovulation during the experimental period. According to Long and Evans (1923), estrus normally occurs 3 to 14 days after littering, when suckling is prevented. Engle and Mermod (1928), in experiments on the prolongation of gestation by anterior pituitary implants, found that rats did not ovulate until after parturition, while Zondek and Ascheim (1928) with one implant, demonstrated ova in the tubes of a mouse which had living fetuses in utero. One of Snyder's rabbits (1934) mated and ovulated on the 46th day while fetuses and necrotic placentas were still in the uterus.

Since the time of ovulation can be determined by the character of the vaginal smear in the rat, this method was used in a number of cases. It was started on the 19th day and continued until the delayed parturition was completed. The type of smear found during pregnancy containing leucocytes, epithelial cells and often simply nondescript cellular material, continued in injected rats as in the normal until the 21st day when mucus and a few red cells were present. The quantity of stringy mucus increased, also the number of red cells and leucocytes. By the 23rd or 24th there was nearly always fresh blood at the mouth of the vagina. This might continue until the 26th day or it might be dark and mingled with mucus as previously noted. Occasionally a smear contained small nucleated epithelial and some cornified cells but there was rarely one of the clear cut estrous character. The most significant results are noted below.

The red cells and leucoyctes were dominant in smears made from no. 169 from the 26th to the 32nd day. Epithelial cells were occasional and few until, on the 29th day, the mouth of the vagina was distended and great numbers of small nucleated epithelial cells were present. The following day there were a few masses of cornified cells. After this leucoyctes were again profuse. In no. 25 the character of the smear changed on the 30th day and cornified epithelial cells were seen along with blood stained mucus and debris. The smear of rat 5 contained cells of the estrous type on the 27th day. She littered on the 27th and 28th and, for several days following, the character of the smear indicated the autolysis of necrotic tissue. On the 39th she came into estrus and mated but did not become pregnant. No. 27 showed cornified cells on the 29th day, though she died

on the 32nd with 4 fetuses in utero. As was noted above, the histological study of the ovaries of nos. 169 and 25 showed atretic follicles but apparently active induced corpora lutea. Correlating the character of the cells in the vagina and the condition of the ovaries, there seems to be an attempt of the organism to resume its normal cycles.

SUMMARY AND CONCLUSIONS

These observations are a further contribution to the physiology of the corpus luteum of the rat. It has been demonstrated that:

1. Pregnancy can regularly be prolonged for 3 or more days by one injection of 75 rat units of human pregnancy urine extract (Antuitrin-S), given on the 19th or 20th day of gestation.
2. Parturition was delayed until the 25th, 26th or 27th day and was also prolonged over 2 or more days.
3. Fetuses remained alive and continued to grow for 2 or 3 days beyond term.
4. Necrosis of retained fetuses began after the 26th day and is indicated by a mucoidal, blood-stained discharge from the vagina.
5. Histological examination of the ovaries revealed induced corpora lutea by the 21st day of pregnancy, but subsequent retrogression of these could not be definitely established by the method used. The induced corpora lutea are regarded as taking over the function of those of pregnancy and their secretion as delaying the onset of labor.
6. Attempts at estrus, as indicated by vaginal smears, were observed as early as the 27th day and are not incompatible with fetuses in utero.

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ON THE RELATION OF DIRECT CURRENTS TO LINEARLY RISING CURRENTS AS STIMULI

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For the purpose of determining the validity of equations devised to represent the excitatory process in tissue it is desirable to compare the results of experiments using stimuli quite different in form. A solution of a particular differential equation might, for example, fortuitously fit data using direct currents as stimuli. That a corresponding integral should also fit data obtained with condenser discharges is very unlikely unless the differential equation represents very closely the mechanism of excitation, the reason for this being that the actual growth curve of the excitatory process will be very different in the two cases because the stimulating currents will be very different at corresponding times. A linearly rising current provides a type of stimulus which differs considerably from direct currents or condenser discharges as regards the values of the currents at corresponding times. It is approximately the opposite in this respect to the condenser discharge so that these two types of stimuli are in a sense complementary as tests of a differential equation designed to represent the mechanism of excitation.

It has already been shown that integrals of the differential equation,

$$\frac{dp}{dt} = KV - kp \qquad (1)$$

where V is the stimulating current or voltage, p is the local excitatory process and K and k are constants, not only adequately represent both time-intensity and voltage-capacity data but that they do so consistently (Blair, 1935). In other words the constant k is the same whether it is derived from direct current data or from condenser discharge data on the same tissue at the same time. It is the present purpose to discuss the application of equation 1 to data of linearly rising currents.

If a current rises linearly at the rate V_0 its value at any time t is V_0t so that equation 1 becomes,

$$\frac{dp}{dt} = KV_0t - kp \qquad (2)$$

If $p = 0$ when $t = 0$ it has been shown already (Blair, 1932b) that the integration of (2) gives,

$$p = KV_0\left[t - \frac{1}{k}(1 - e^{-kt})\right] \tag{3}$$

If p, to be adequate, must attain a value h, equation 3 becomes for adequate stimuli,

$$\frac{kh}{K} = V_0\left[t - \frac{1}{k}(1 - e^{-kt})\right]$$

or since, as t becomes very large V_0 becomes very small and V_0t becomes the rheobase R, $\frac{kh}{K} = R$

$$V_0t - R = \frac{V_0}{k}(1 - e^{-kt}) \tag{4}$$

Usually, however, the threshold is not a constant h but depends (according to the relation $p = h \pm \alpha V$ where α is a constant) on the value of the stimulus at the moment p becomes adequate. This gives instead of equation 4,

$$V_0t - R = \frac{K}{K \mp k\,\alpha}\left[\frac{V_0}{k}(1 - e^{-kt})\right] \tag{5}$$

Equation 5 should, according to the present point of view, always represent time-intensity curves with linearly rising currents and equation 4 should do so in the particular case $\alpha = 0$.

These equations alone, on account of their forms, are not easily applied. If, however, the constant k is derived from direct current data obtained at the same time the quantities $Q = 1 - e^{-kt}$ can be evaluated separately. When this has been done $V_0t - R$ when plotted against V_0Q should give a straight line of slope $\frac{1}{k}$ in the case of equation 4 and of slope $\frac{K}{k\,(K \mp k\,\alpha\,)}$ in the case of equation 5.

In the former case there would be no arbitrary constant at all since k was separately derived and in the latter case there would be the single arbitrary constant $\frac{K}{k\,(K \mp k\,\alpha\,)}$. This constant, however, is obtainable also from direct current data as will now be shown.

The solution (Blair, 1932a, c) of equation 1 for direct currents with $p = h \pm \alpha V$ for adequacy is

$$\log \frac{V}{V - R} = kt + C \tag{6}$$

where C is $-\log \frac{K}{K \mp k\,\alpha}$. Therefore direct current data on the same tissue at the same time provide both the constant k and the quantity $\frac{K}{K \mp k\,\alpha}$. When these values have been obtained equation 5 may be tested easily as there are no arbitrary constants remaining. The present paper deals with such a test of equation 5.

Apparatus and method. The apparatus for obtaining rectangular waves for the direct current measurements has been described previously (Blair, 1935). It consists essentially of a hard rubber disc 30 cm. in diameter fixed on a similar brass disc which is mounted so that it may be driven by a synchronous motor. In the face of the rubber disc is a brass wedge with its base toward the center and its apex toward the periphery of the wheel. Two brushes of lubricated carbon are held in a movable carriage so that they bear on the rubber disc or on the brass wedge. When both are on the brass wedge the circuit is completed for a duration which is relatively long near the center of the disc and short near the periphery for any given speed of rotation.

For obtaining currents of linear rise an inductance of about 50 henries with a resistance of 2,000 ohms is used. The time constant of this circuit is $\frac{1}{40}$ second. This is the time required for the current to reach about two-thirds of its final value. The current rises linearly in rough approximation during this time. The greatest durations actually used, however, are less than one-third of this so that the currents obtained are linear in very close approximation. In the actual arrangement the battery, the inductance, a 1000 ohm resistor, a switch and the brushes previously described are connected in series. A potentiometer arrangement in parallel with the 1000 ohm resistor is used to deliver current to the tissue. While the disc is running a current of linear rise will pass through the circuit on each contact of the brushes with the wedge as long as the switch is closed. The rate of rise of the current in the main circuit is, of course, always the same. The rate of rise of the current through the tissue, however, is in direct proportion to the fraction of the potentiometer resistance being used. Thus for any particular duration determined by the setting of the brushes with the disc it can be ascertained what rate of rise, as determined by the potentiometer adjustment, is necessary to provide an adequate stimulus. The product of this duration and the rate of rise gives a measure of the terminal value of the stimulating current, the quantity V_0t of equation 5.

With such a large inductance as that being used there is, of course, a great deal of energy in the magnetic field of the circuit at the moment it is broken. Consequently the current will continue to flow with sparking across the switches unless this energy becomes dissipated in some other

way. There is included, therefore, in the circuit a Thyratron tube in parallel with the inductance alone, the grid to cathode connection being used so that the tube will start easily. This arrangement effectively takes up the surge from the inductance and the current through the potentiometer stops abruptly giving a wave of linear rise and perpendicular fall.

If it is desired that the current should not cease abruptly the Thyratron may be connected across the potentiometer in addition. In this case, if the resistances of the battery and the Thyratron are similar, the current in the potentiometer will decay with a time constant about equal to that with which it rose. This arrangement was not used in the present work.

In order that comparable measures may be made of the intensities of the two kinds of stimuli it is necessary that the terminal voltages of linear rise and the direct current voltages may be put on the same scale. This is accomplished by placing an ohmic resistance in parallel with the inductance so that by means of a switch the current may be sent through either the one or the other. The resistance is then adjusted so that it is equal to the impedance of the inductance for some particular duration, the adjustment of equality being made by observing the deflection of an oscillograph or an ammeter when the circuit is being made and broken rapidly, first through the resistance and then afterward through the inductance. If an ammeter is used its reading with the rectangular wave through the resistance will be double the triangular wave through the inductance when the peak voltages and as a consequence the resistance and the impedence are equal. In the present case the adjustment is made to give equality at about 0.002 second. The currents from the inductance are sufficiently linear so that they diverge from equality by only 3 per cent at 0.007 second.

In all cases sciatic-gastrocnemius preparations of the frog were used. The nerve was excited and the muscle used to indicate the adequacy of the stimuli. Chlorinated silver wires 0.5 mm. in diameter were used as electrodes, the nerve having been suspended upon them in air. The separation of the electrodes was different in different experiments. The data for the two types of stimuli were obtained in succession as quickly as possible in order that the tissue might not change appreciably during the experiment. All the measurements were made at room temperatures except for two sets at 6°C.

Experimental results. As it is to be expected on general grounds as well as from the experiments of Lapicque (1926) with double condensers, the currents of linear rise give a *terminal voltage-time of rise* curve similar to the direct current *time-intensity* curve provided the rate of rise is not too small. When the rate of rise is less than a certain value the curves differ in that the terminal voltages begin to increase again instead of tending to approach a rheobase. This latter phenomenon which was observed very

early (*see* Lapicque, 1926) was studied considerably by Keith Lucas (1907). By allowing currents to rise linearly to certain steady values, he showed that the rheobase was no longer adequate if not reached within a certain time and also, that if the rate of rise was made less than a certain value no terminal voltage of the usual order of magnitude would excite. The present measurements deal with the events occurring in the times shorter than these. In table 1 are given the data of a typical experiment. In the first column are the times of rise in milli-seconds. In the second are given the rates of rise, V_0, on an arbitrary scale. The third column contains the products of the rates of rise and times of rise, V_0t, or the terminal

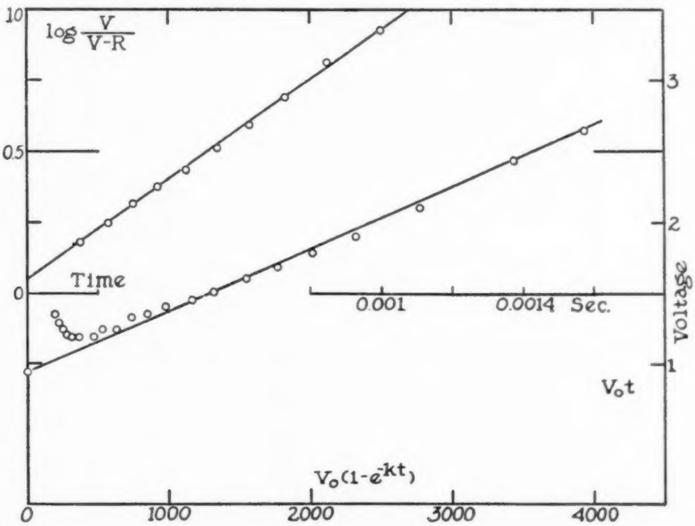

Fig. 1

voltages, which are also arbitrary. In the fifth column are the direct current voltages, V, required for the durations of the first column. These are on the same scale as V_0t. The sixth column gives the quantities $\log_{10} \frac{V}{V-R}$ of equation 6. The fourth column contains the quantities $V_0(1 - e^{-kt})$ of equation 5 which were calculated from the direct current data by a method which can be followed by reference to figure 1.

In the upper part of figure 1 are plotted the quantities $\log \frac{V}{V-R}$ of table 1 as ordinates against the corresponding durations as abscissae. According to equation 6 these should give a straight line of slope k with

an intercept C on the ordinate. The slope of the line as drawn is 875 approximately, which is k. Since, however, logarithms to base 10 are used this number must be multiplied by 2.303 giving 2010 approximately as the proper k. By using this k and the proper durations t the quantities V_0 $(1 - e^{-kt})$ of column 4 may be calculated.

TABLE 1

Data of direct and linearly rising current time-intensity curves at 22°C.

DURATIONS	RATE OF RISE V_o	TERMINAL VOLTAGE $V_o t$	$V_o (1 - e^{-kt})$	D. C. VOLTAGE V	LOG $\frac{V}{V - R}$
milli-seconds					
6.95	194	1.35		0.93	
5.75	223	1.28			
4.95	252	1.24			
4.28	282	1.21			
4.15				0.94	
3.72	320	1.19			
3.26	363	1.19			
3.02				0.94	
2.56	468	1.19			
2.25				0.95	
2.21	530	1.24			
2.03					
1.94	635	1.24			
1.74	763	1.33	740	0.96	
1.52	892	1.36	850		
1.35	1056	1.42	985	0.98	
1.14	1285	1.46	1160		
1.00	1523	1.52	1320	1.06	0.931
0.85	1887	1.61	1550	1.11	0.818
0.73	2300	1.68	1770	1.18	0.688
0.63	2810	1.78	2010	1.26	0.592
0.54	3520	1.90	2320	1.36	0.511
0.45	4670	2.11	2770	1.49	0.433
0.37	6580	2.43	3440	1.62	0.375
0.30	8770	2.64	3940	1.80	0.318
0.23	>11000			2.14	0.248
0.15				2.76	0.179

$k = 2010$

$\frac{K}{K + k\alpha} \times \frac{1}{k} = \frac{1}{2250}$

The intercept C, in this case 0.05, is equal to $-\log \frac{K}{K \mp k\,\alpha}$. Therefore the quantity $\frac{K}{K \mp k\,\alpha}$ of equation 5 is $\frac{1}{1.12}$. If now, according to equation 5, $V_0 t$ is plotted against V_0 $(1 - e^{-kt})$ a linear relation should appear and

the slope of the line should be $\frac{K}{K + k\,\alpha} \times \frac{1}{k} = \frac{1}{1.12} \times \frac{1}{2010} = \frac{1}{2250}$. This line should also cut the ordinate, V_0t, axis at the rheobase R.

In the lower part of figure 1 are plotted V_0t against $V_0\,(1 - e^{-kt})$ and the rheobase is marked on the ordinate. It will be seen that the right hand side of the graph is linear according to the prediction of equation 5 but the left hand side is not. The slope of the line drawn is $\frac{1}{2320}$ which is close to the predicted value $\frac{1}{2250}$ and the intercept is at the rheobase. It

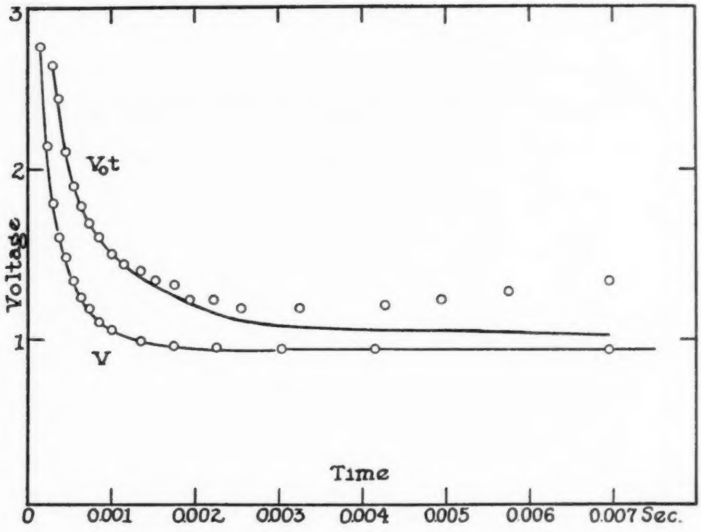

Fig. 2

appears, therefore, that equation 5 is valid for the rapid rates of rise but not for the slow.

Since the durations themselves do not appear in figure 1 a better idea of the range of validity of equation 5 can be obtained from the time-intensity curves of both the direct and linearly rising currents which are given in figure 2. In this figure are plotted V and V_0t as ordinates and the corresponding durations as abscissae giving the direct current time-intensity curve, lower, and the *terminal voltage-time of rise* curve, upper, for the linearly rising currents. In addition there is drawn between the two the theoretical curve as predicted by equation 5 and given by the straight line in figure 1.

It will be seen that the linear rise curve is similar to the time-intensity curve for the short durations but that as the time of rise increases the terminal voltage goes through a minimum and then rises again instead of approaching asymptotically the direct current rheobase. The greatest durations are not long enough to reach the limiting slope found by Keith Lucas.

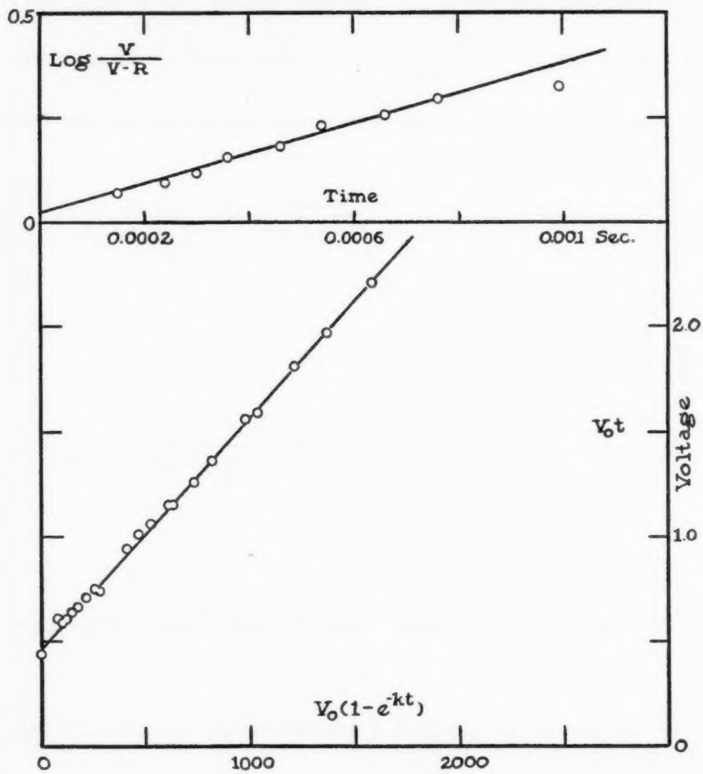

Fig. 3

The data conform to equation 5 until the durations become about equal to that at which the time-intensity curve has reached the rheobase within a few per cent, i.e., at about 1.5 milli-second in the case of figure 2. The divergence of the data from the theoretical curve is still less than 10 per cent at 3 milli-seconds. This case is typical qualitatively of all the preparations studied. The time at which the divergence occurs is not

constant, but depends on the same things to some extent as the excitability. This is illustrated in figures 3 and 4.

In these figures are plotted the same kind of data as those in figures 1 and 2. The data are given in table 2. The preparation in this case was used at 6°C. and the excitability is less. It will be seen from figure 3 that k and C are both much less in this preparation than the former. Since $k = 805$ to base e and $\frac{K}{K + k\,\alpha} = \frac{1}{1.06}$ the predicted slope of the lower graph is $\frac{1}{850}$ approximately. The line drawn has a slope $\frac{1}{890}$ approxi-

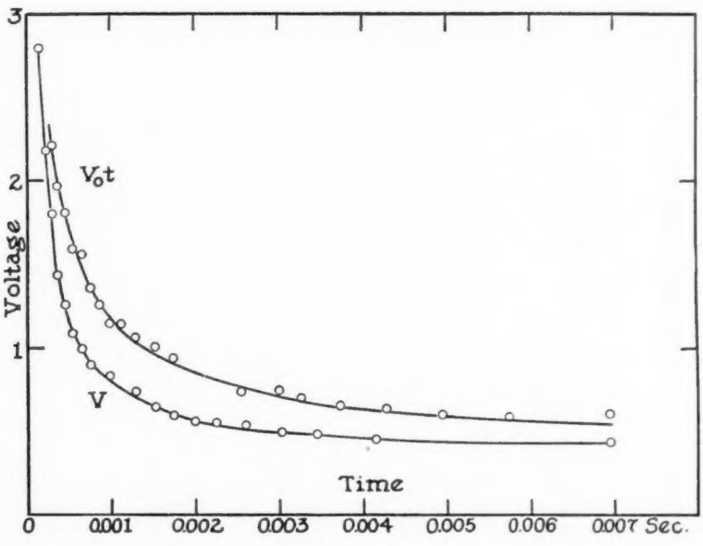

Fig. 4

mately. Therefore in this case also the linear rise data conform quite well to equation 5 and the divergence at the left-hand side of the graph with the slowest rates of rise is much less. The reason for this is evident from figure 4 which shows that the divergence of V_0t from the theoretical curve occurs much later in actual time but at about the same physiological time since the direct current curve approaches much more slowly its rheobase. The final elevation of the terminal voltage with slow rates of rise is delayed since in this case there is no great increase of V_0t even at the longest duration used.

In table 3 are given the results of sixteen experiments similar to those already discussed. The first column gives the distance between the

stimulating electrodes and the second gives the age of the preparation after dissection. In the third column are the k values using logarithms to base e obtained from graphs such as figure 1 upper, and the next column gives the quantities $\frac{K}{K + k\,\alpha}$ similarly obtained. The fifth column is the recip-

TABLE 2

Data of direct and linearly rising current time-intensity curves at 6°C.

DURATIONS	RATE OF RISE V_o	TERMINAL VOLTAGE $V_o t$	$V_o\,(1 - e^{-kt})$	D. C. VOLTAGE V	LOG $\frac{V}{V - R}$
milli-seconds					
6.95	89	0.62		0.43	
5.75	103	0.59			
4.95	124	0.61			
4.28	149	0.64			
4.15				0.46	1.322
3.72	179	0.67			
3.45				0.48	1.041
3.26	217	0.71	204		
3.00	251	0.75	229		
2.56	288	0.74	255		
2.25				0.56	0.678
1.99				0.57	0.635
1.74	538	0.94	405	0.60	0.571
1.52	668	1.02	470	0.65	0.487
1.29	825	1.07	528	0.74	0.391
1.12	1030	1.15	610		
0.98	1165	1.15	635	0.83	0.324
0.86	1460	1.26	730		
0.76	1800	1.37	820	0.91	0.288
0.66	2370	1.56	975	0.99	0.255
0.54	2940	1.58	1035	1.08	0.228
0.46	3940	1.81	1210	1.27	0.182
0.36	5450	1.97	1370	1.43	0.158
0.30	7340	2.21	1580	1.80	0.121
0.22				2.18	0.097
0.15				2.80	0.072

$k = 805$

$\frac{K}{K + k\alpha} \times \frac{1}{k} = \frac{1}{850}$

rocal of the product $\frac{1}{k} \times \frac{K}{K + k\,\alpha}$ or the reciprocal of the predicted slope of the linear parts of graphs like figures 1 and 3 lower. The sixth column gives the reciprocals of the measured slopes of these same lines. The last column gives the ratios of the theoretical to the measured slopes.

It will be seen that these latter ratios diverge considerably from unity in some cases but this is to be expected, since the arbitrary constants are derived from the separate direct current data and the tissue is likely to vary somewhat between the measurements. The average value is very close to unity, however, indicating that there is no systematic divergence. This indicates in turn that equation 5 is valid for the currents of linear rise providing they do not rise too slowly. From figures 1 and 3 it will be seen that the current must rise at a rate at least as fast, approximately, as one rheobase in six chronaxies in order to be fast enough to satisfy the equation.

TABLE 3

The relation of the direct current to the linear rise constants

SEPARATION	AGE	k	$\frac{K}{K + k\alpha}$	PREDICTED RECIPROCAL SLOPE	MEASURED RECIPROCAL SLOPE	$\frac{\text{PREDICTED}}{\text{MEASURED}}$
mm.	*days*					
11	1	1530	1	1530	1540	0.99
10	1	1640	1	1640	1715	0.95
22	$\frac{1}{6}$	1725	1/1.12	1930	1650	1.17
25	1	2645	1/1.45	3842	4350	0.88
14	1	3200	1/1.50	4800	4900	0.98
14	1	2250	1/1.17	2630	2700	0.98
15	2	2300	1/1.74	4000	3400	1.17
15	7	2300	1/1.41	3240	3130	1.03
10	7	2370	1/1.26	2980	3400	0.88
10	1	1740	1/1.17	2040	1925	1.06
10	1	1840	1/1.26	2320	2320	1.00
6	1	3020	1/1.78	5380	5000	1.07
13*	$\frac{1}{6}$	1150	1/1.06	1220	1450	0.84
13**	$\frac{1}{4}$	805	1/1.06	850	890	0.95
10	2	2920	1/1.68	4905	4555	1.07
17***	1	2010	1/1.12	2250	2270	0.99
Average						1.006

All measurements at room temperature (about 22°C.) except preparations * and ** made at 6°C.

** Summary of data from table 2.

*** Summary of data from table 1.

On general grounds it is very unlikely that the point at which equation 5 fails indicates the entrance of a new reaction. Rather it is likely that this effect enters at zero time but is negligibly small during ordinary time-intensity curve durations. There is no evidence in the present data to indicate the cause of this rise of the terminal voltages but it is well known that the current in tissue due to a constant voltage decreases with time due to polarization and there appears to be no reason at present for introducing any other hypothesis in explanation. It is therefore assumed that

if the actual effective current could be measured the data so obtained would conform to equation 5 to a considerably greater extent at least than they now do.

It must be assumed that, for the same reason, the direct current rheobase is also somewhat raised in general above its theoretical value. On the other hand so must all the other direct current intensities be similarly somewhat raised, i.e., the direct current time-intensity curve will be shifted upward somewhat, more near the rheobase than with the shorter times but probably not sufficiently more, considering the limit of accuracy of the measurements, to be given consideration. With slowly rising stimuli, however, this can no longer be expected to be true when they become of such magnitude that they cause greater polarization than does the direct current rheobase during its utilization time.

Relation to other work. It has already been mentioned that Lapicque's double condenser arrangement gives a curve similar to the linear rise curve in figure 2. It is mathematically extremely difficult, however, to deal with this type of stimulus so that an exact comparison will not be attempted.

Keith Lucas's (1907) most rapidly rising currents required 0.03 second to attain their peak values. His work therefore lay in a time range far beyond the time-intensity curves of the tissues used, namely, the sciatic nerves of the frog and of the toad and the sartorius muscle of the toad. With the nerves he found that the rheobase had to be exceeded by only 50 to 100 per cent even with these slow rates of rise. In the present work one week-old preparation rose to twice the rheobase at 0.007 second although as a rule the divergence was of the order of that in figure 2. Lucas, however, worked at about 10°C.

With the toad's muscle about 15°C. he found that the rheobase was adequate whether attained immediately or after a linear rise of 0.09 to 0.1 second. With slower rates of rise the current had to be raised as with the nerve. Since the time-intensity curves of the muscle and the nerve are similar it seems probable from these results that equation 5 would fit muscle data over a much greater range of time. Also it may be inferred that if this phenomenon is a physiological reaction rather than something due to the simple decay of the effective current it is not related in muscle to the excitatory process proper in the same way as it is in nerve.

In using alternating currents as stimuli an optimum frequency is observed (e.g., Achelis, 1930). This optimum frequency depends on the temperature and is lower at lower temperatures. Referring to figure 2 it would be expected that an optimum alternating current stimulus would occur at about the minimum of the linear rise curve, i.e., at about 3.5 milli-seconds. An alternating current stimulus of frequency $\frac{1000}{4 \times 3.5} =$ 70 approximately, would rise to its maximum voltage in this time. Achelis

gives optimal frequencies of 54 and 210, respectively, at 15° and 25°C. for the sciatic nerve of the frog. The temperature of the figure 2 preparation lay between these. According to figure 4 the optimal frequency at 6°C. would be much lower since the minimum is at about 0.006 second corresponding to an alternating current frequency of 40 approximately.

It is to be expected that the same phenomenon which is observed with slowly rising currents will also be a factor in experiments on the addition of inadequate stimuli of the type in which the first is prolonged. These have been studied extensively by Bishop (1928) and Erlanger and Blair (1931).

In Bishop's work brief test shocks were added to constant currents slightly sub-rheobasic at different times up to about 0.004 second after the beginning of the latter stimuli. His results are given (fig. 4, 1928a) in graphic form indicating a relation

$$\log (\text{test shock}) = \text{constant} \times \text{interval} + \text{constant}.$$

This is the type of relation which is predicted from equation 1 for the addition of such stimuli unless the constant current is not quite near the rheobase. If the constant current is more than slightly sub-rheobasic a curve downward such as Bishop's graphs show sometimes at long times is to be expected.

Bishop's results can be taken to indicate that no long time effects were present in measurable amount up to 0.004 second because if they were his lines would curve up at long times instead of down.

The work of Erlanger and Blair (1931) was similar but was extended to much longer times. They found that the shock strength diminished from zero interval up to about 0.002 second, the exact time depending upon the temperature and being longer at lower temperatures. At greater intervals the shock strength had to be increased again. The phenomenon giving rise to this increase is evidently of the same kind as that which necessitates the increase of the terminal voltage with slowly rising current.

Bishop (1928b) gives a physical analogy to account for this phenomenon in the form of two leaky condensers in series. If the excitatory process is proportional to the charge of the smaller condenser or to the magnitude of the current through its leak, it will, on the application of a steady electromotive force, attain a value greater than its final at some time previous to reaching the steady state in which both condensers are charged to equilibrium. The large capacity in this analogy is said to correspond to structures other than that directly excitable. Whatever may be the virtues of this analogy, which are difficult to evaluate on account of the forms of the equations, it is derived by Bishop from experimental evidence leading him to the conclusion that the effective stimulating current is not constant but is

reduced by polarization other than the excitatory process itself. Qualitatively nothing else is required in explanation of prolonged stimuli.

It does not appear to have been demonstrated that these polarizations are of the proper relative speeds to account for these long time effects in tissues of lower excitabilities. As has been known for a long time the greatest time which may be used to attain an effective rheobase is much greater with lower excitabilities than with high. Fick (Lapicque, 1926) gives 10 seconds with *Anodonta* which must be at least 1000 times as great as the corresponding permissible duration in the frog. Observations (Lapicque, 1926; Lucas, 1907) of this kind indicate that the influence of the prolonged currents enter at about corresponding physiological times whether those are very short in tissues of high excitability or very long in tissues of low excitability. It may therefore be expected that equation 5 will be valid to about the same physiological time in all tissues.

Conclusions. Whatever may be the cause of these effects at longer times it seems possible to conclude that equations 1 and 5 are valid in close approximation for ordinary physiological ranges if the action current is the primary factor in the transmission of the nervous impulse. The action current in form is intermediate to the current of linear rise and the rectangular wave. Its rising phase is given as 0.3 milli-second by Gasser and Erlanger (1927) for frog's nerve in the excitability range being included here. Therefore the action potential will ordinarily stimulate the succeeding inactive section of the nerve in a time considerably shorter than that at which the linearly rising current curves diverge from their predicted values.

Whether these long time effects are due primarily to purely physical causes or to a physiological mechanism such as the active inhibitory process proposed by Rashevsky (1933) or to the using up of excitatory substance as suggested by Hill (1910) it seems permissible to conclude that the excitatory process as represented by equation 1 is the one which is of primary physiological importance. It has now been shown for the frog's sciatic nerve that the constant k is the same, in close approximation at least, whether it is derived from direct current, or condenser, or linearly rising stimuli. In addition (Blair, 1934a), these constants derived from excitation data appear to be consistent with the velocity data of the same fibres. Separate time-intensity and voltage-capacity data of other tissues (Blair, 1932a) conform to the predictions of equation 1 so that its application is probably quite general. Since this equation may equally well describe a number of different physico-chemical mechanisms it is useless at present to attempt to give it a precise meaning. A systematic study of the variations of the factor k in a particular tissue under the influence of different reagents whose effects are at least partially known should, however, eventually permit a choice of mechanisms to be made.

SUMMARY

A group of time-intensity curves using currents of linear rise and perpendicular fall are related to the direct current time-intensity curves for the same tissues (sciatic-gastrocnemius preparations of the frog) at the same time. It is shown that solutions of the differential equation,

$$\frac{dp}{dt} = KV - kp$$

where p is the local excitatory process, V the applied potential and K and k are constants, are consistent in that the constants are the same whether derived from the one type of data or the other. The time-intensity curve of linear rise goes through a minimum in agreement with older work on slowly rising currents.

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THE METABOLIC FATE OF GALACTOSE IN ADULT DOGS AND RABBITS

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The fate of galactose in separate tissues of the animal body is a question that has received little attention. In any extensive study of the metabolism of this carbohydrate, consideration of this question assumes importance. This paper is a report of observations bearing on this problem.

THE CONVERSION OF GALACTOSE INTO GLUCOSE. Harding and Van Nostrand (1) and Harding and Grant (2) have reported evidence indicating that, following absorption from the alimentary tract, galactose is converted into glucose in normal human subjects. Roe and Schwartzman (3) did not obtain evidence of galactose conversion into glucose in normal human subjects, but their data show uniform and striking increases in the fermentable sugar of the blood of diabetic patients after oral administration of galactose. Cori and Cori (4) did not find an increase in the blood glucose of rats during galactose absorption. When galactose was fed to rats, in doses of 0.6 to 1.2 grams per kgm. of body weight, by Harding, Grant and Glaister (5), the conversion of galactose into glucose was not observed. Blanco (6) administered galactose orally, subcutaneously, and intravenously, in doses of 2 grams per kgm. of body weight, to amytalized rabbits that had not been fasted, and obtained data showing increases in the fermentable reducing substance of the blood after each type of galactose administration. Following the injection of galactose into fasting rabbits, Corley (7) did not obtain significant increases in the fermentable reducing substance of the blood.

Since the evidence for the conversion of galactose into glucose in animals was found to be contradictory, and somewhat in disagreement with the data obtained upon human subjects, it was decided to make some studies upon this fundamental question.

Experimental. Both fasted and non-fasted rabbits were used in these

[1] The data reported in this paper are taken from a dissertation submitted by Joseph H. Roe to Yale University, May, 1934, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

experiments. Blood was collected from the marginal ear vein. Control samples of blood were taken and galactose was administered by mouth in some experiments and intraperitoneally in others, the dosage being 5 grams per kgm. of body weight. Samples of blood were then drawn at intervals for a period of 4 hours after administration. The blood galactose was determined by the method of Roe and Schwartzman (3) and the total blood sugar was estimated by Benedict's (8) procedure. As these two methods of analysis involve a copper reduction procedure upon blood filtrates, the one being applied before and the other after yeast fermentation of the blood, it is readily possible to calculate the glucose present in a blood sample from the data obtained.

Results. The results of these experiments are shown in table 1. Rabbits 1 and 2 were fasted for 40 hours before starting the experiment and galactose was administered to them by mouth. There was an appreciable increase in the blood galactose of these animals following galactose ingestion, but the blood glucose remained at essentially the same level. There is no indication of a transformation of galactose into glucose in the data obtained upon these two rabbits.

Rabbits 3 and 4 were not fasted, and galactose, in an approximately isotonic solution warmed to 38°C., was injected intraperitoneally. A marked increase in the glucose of the blood of both of these rabbits occurred following galactose administration. The results of the experiments with these two rabbits thus seem to indicate that galactose is transformed into glucose in the rabbit and that this transformation may be readily demonstrated in rabbits that have not been fasted.

The usual procedure was first carried out upon rabbits 5, 6 and 7 without fasting them, and then about a week later the same experimental procedure was repeated upon these animals after they had been fasted for 46 hours. In all of these experiments the galactose was administered intraperitoneally. In the experiments in which the animals had been fasted the data do not indicate any conversion of galactose into glucose and, in fact, there was some decrease in the blood glucose level; but in each experiment in which the animals had not been fasted a very definite increase in blood glucose was observed following galactose administration. Thus in the same animal it was shown that an increase in the blood glucose occurred after galactose injection when the animal had been fed up to the time of the experiment, and that such an increase could not be demonstrated if the animal had been fasted.

It is of interest also that in rabbits 5, 6 and 7, when fasted, a much greater galactemia occurred after galactose administration, which probably was not due to a more rapid absorption, because the method of administration was intraperitoneal injection. From this it appears that the rate of metabolic conversion of galactose is slower in the fasted organism.

The results of these experiments seem to indicate that, following the administration of galactose into rabbits, a conversion of galactose into glucose occurs, which is demonstrable only in non-fasted animals. Our results confirm the findings of Blanco (6), who used rabbits which were fed, and also the data of Corley (7), obtained upon fasted animals.

TABLE 1

The conversion of galactose into glucose

The galactose and glucose content of the blood of rabbits after galactose administration. Dose, 5 grams per kgm. of body weight. To rabbits 1 and 2 the galactose was administered by mouth; to rabbits 3, 4, 5, 6, and 7, intraperitoneally.

RABBIT	HOURS FASTED	SUGAR	MGM. PER 100 CC. OF BLOOD AS GLUCOSE				
			Minutes after administration				
			0	40	90	150	240
1	40	Galactose	—	104	205	244	244
		Glucose	72	66	71	76	82
2	40	Galactose	—	146	252	292	296
		Glucose	71	60	71	70	66
3	0	Galactose	—	171	245	235	194
		Glucose	—	79	106	133	115
4	0	Galactose	—	91	154	172	187
		Glucose	—	85	111	137	155
5	0	Galactose	—	151	144	88	28
		Glucose	72	83	84	78	114
	46	Galactose	—	465	536	442	416
		Glucose	80	37	38	42	58
6	0	Galactose	—	238	225	143	74
		Glucose	80	80	74	96	109
	46	Galactose	—	430	457	357	296
		Glucose	103	48	51	67	74
7	0	Galactose	—	161	166	125	69
		Glucose	65	79	82	98	148
	46	Galactose	—	567	640	584	491
		Glucose	68	48	31	43	57

THE FATE OF GALACTOSE IN MUSCLE AND NERVE TISSUE. To determine whether galactose is utilized by muscle and nerve tissue, experiments were planned which consisted of the determination of the galactose content of the afferent and efferent blood to the leg and the brain of the dog following galactose administration.

Experimental. The dogs used were fasted over night previous to each experiment. The galactose was administered intraperitoneally in some experiments and subcutaneously in others. The dosages used ranged from 1 to 5 grams per kgm. of body weight. Nembutal and ether were used as anesthetics. At varying intervals following the administration of galactose, samples of blood were collected simultaneously from the femoral artery and vein in the experiments upon muscle tissue, and from a common carotid artery and an internal jugular vein in the experiments upon nerve tissue. These samples were analyzed for galactose by the method of Roe and Schwartzman (3).

TABLE 2

Arterial and venous blood galactose concentrations in dogs following the administration of galactose

Different dogs were used for the muscle and nerve tissue experiments. The data are grouped together to save space. The time of collection of the carotid-jugular samples was approximately as indicated in the minutes after administration column.

EXPERIMENT	MINUTES AFTER ADMINISTRATION	GALACTOSE					
		Mgm. per 100 cc. of blood		Per cent change	Mgm. per 100 cc. of blood		Per cent change
		Femoral artery	Femoral vein		Carotid artery	Jugular vein	
1	25	150	127	−15.3	59	47	−20.3
	45	133	123	−7.5	95	87	−8.0
	65	94	94	0	68	68	0
	85	88	90	+2.2			
2	25	171	114	−33.3	52	57	+9.6
	45	260	203	−21.9	95	95	0
	65	240	227	−5.4			
	85	248	236	−4.8			
3	50	49	51	+4.0	38	41	+7.8
	65	58	58	0	46	46	0
	80	60	56	−6.6			
	95	46	42	−8.7			
4	75	121	129	+6.2	165	172	+4.0
	90	120	119	−0.8	114	121	+5.7
	105	62*	68*	+8.8			
	120	55*	63*	+12.7			

*** These samples were collected after exercising the leg by stimulating with single induction shocks at the rate of 1 per second for 15 minutes.**

Results. The results of these experiments are shown in table 2. The differences in the galactose content of the simultaneously collected pairs

of blood samples, which were drawn 50 minutes, or more, after galactose administration, are essentially within the limits of experimental error. The variations are also both positive and negative and thus there is not shown any tendency towards a change in concentration in either direction. In experiments 1 and 2, however, there is an interesting difference in the galactose content of the arterial-venous pairs of blood samples collected early in the experiment. Thus, in the pairs collected 25 and 45 minutes after galactose administration the venous blood of the leg showed decreases in galactose content of 15 to 33 per cent; and in experiment 1, the carotid-jugular samples of blood collected 25 minutes after galactose administration, reveal a 20 per cent diminution in the galactose content of the venous blood. These differences are greater than the experimental error of the method used and must be recognized as significant withdrawals of galactose.

These results seem to suggest that galactose is neither oxidized, nor converted into glycogen, in voluntary muscle tissue, and is not oxidized in nerve tissue. The positive withdrawals of galactose from the blood early in experiments 1 and 2 are believed to have been due to the preliminary active absorption of galactose by the tissues. As galactose is fairly soluble and readily diffusible, it is reasonable to assume that additional amounts of galactose would be absorbed by the tissues early in the experiment until an equilibrium is established. In 1922 Folin and Berglund (9) called attention to the importance of the tissues as reservoirs for the withdrawal of foreign sugars from the blood. The data obtained early in our experiments are in accord with the suggestion of these authors. On the other hand, all of our results obtained 50 minutes, or more, after the administration of galactose show no significant withdrawals of galactose from the blood which passed through the leg and the brain of the dog.

Harding and Grant (2) found less galactose in the venous blood than in the arterial blood of human subjects receiving 40 grams of galactose by mouth. The arterial-venous differences in these authors' experiments are small, however, and certainly are not of the magnitude obtained when glucose is fed. Harding and Grant also used relatively smaller doses of galactose than were used in our experiments and therefore the lower venous blood concentrations of galactose obtained in their experiments might be attributed to the "tissue absorption" which apparently occurred in the early part of some of our experiments.

There are two reports in the literature of perfusion experiments in which it was attempted to show whether galactose is utilized by muscle tissues. In 1908 McGuigan (10) perfused the leg of a dog with a galactose solution and observed a significant decrease in the sugar content of the perfusion fluid. Griesbach (11), in 1929, obtained results exactly opposite to those of McGuigan. The results of our experiments confirm the work of Griesbach.

TABLE 3

The rôle of the liver in galactose metabolism

The galactose, total sugar, and glucose content of blood samples collected simultaneously from the portal and the hepatic veins following the injection of galactose into the duodenum. No galactose was administered to rabbits 6 and 7, which are controls upon the experimental procedure.

RABBIT	MINUTES AFTER INJECTION	VEIN	MILLIGRAMS PER 100 CC. OF BLOOD AS GLUCOSE				
			Galactose		Total sugar	Glucose	
			Total	Change		Total	Change
1	0	Portal				151	
		Hepatic				172	+21
	30	Portal	226		470	244	
		Hepatic	151	−75	500	349	+105
	60	Portal	385		604	219	
		Hepatic	195	−190	435	240	+21
2	0	Portal				94	
		Hepatic				111	+17
	30	Portal	261		454	193	
		Hepatic	184	−77	425	241	+48
	60	Portal	350		662	312	
		Hepatic	175	−175	592	417	+105
3	0	Portal				126	
		Hepatic				157	+31
	30	Portal	93		272	179	
		Hepatic	98	+5	355	257	+78
	60	Portal	263		526	263	
		Hepatic	127	−136	460	333	+70
4	0	Portal				120	
		Hepatic				146	+26
	30	Portal	149		418	269	
		Hepatic	117	−32	410	293	+24
5	0	Portal				107	
		Hepatic				123	+16
	30	Portal	118		335	217	
		Hepatic	103	−15	352	249	+32
	60	Portal	152		448	296	
		Hepatic	133	−19	477	344	+52
6	0	Portal				61	
		Hepatic				69	+8
	30	Portal				93	
		Hepatic				100	+7
	60	Portal				69	
		Hepatic				74	+5

TABLE 3—*Concluded*

RABBIT	MINUTES AFTER INJECTION	VEIN	MILLIGRAMS PER 100 CC. OF BLOOD AS GLUCOSE				
			Galactose		Total sugar	Glucose	
			Total	Change		Total	Change
7	0	Portal				86	
		Hepatic				109	+23
	30	Portal				117	
		Hepatic				144	+27
	60	Portal				130	
		Hepatic				144	+14

THE RÔLE OF THE LIVER IN GALACTOSE METABOLISM. *Experimental.* An experimental procedure was developed to study the sugar composition of the afferent and efferent blood to the liver, before and after galactose administration. Rabbits were fasted over night. Light anesthesia was produced by injecting intraperitoneally 30 mgm. of nembutal per kilogram of body weight and at the end of 30 minutes surgical anesthesia was established by the use of ether. After complete anesthesia had been established, a longitudinal slit was made in the rabbit's abdomen, and samples of blood were collected simultaneously from the portal vein and from one of the hepatic veins. After collecting these samples of blood to serve as controls, galactose in 30 per cent solution was injected into the duodenum by means of a hypodermic syringe. The dose of galactose was 5 grams per kgm. of body weight. The rabbit's abdomen was then closed by means of clamps and the animal was kept moderately warm with an electric heater. Simultaneously collected samples of blood were again taken from the portal and hepatic veins 30 and 60 minutes after the injection of galactose into the duodenum. The blood samples were analyzed for galactose and total sugar; the glucose was calculated from these data.

Results. The results of these experiments are shown in table 3. The analyses of each simultaneously collected pair of blood samples, except one, show a lower concentration of galactose in the blood of the hepatic vein, the withdrawals of galactose from the blood which passed through the liver ranging from 15 to 190 mgm. per 100 cc. These changes are of a magnitude that makes their interpretation independent of allowance for experimental error. An examination of the data will show that the galactose was increasing in the blood of the portal vein, by absorption from the intestinal tract, at the rate of 3 to 6 mgm. per 100 cc. per minute. It requires less than 1 minute for the blood entering by the portal vein to pass through the liver. We should therefore expect less than 6 mgm. per 100 cc. differences in the galactose concentrations of the pairs of blood samples due to the fact that the bloods were collected simultaneously. It must be con-

sidered also that some of the afferent blood to the liver enters through the hepatic artery and the blood of this artery may have a diluting effect upon the galactose concentration of the portal blood. But this diluting effect is slight, since the blood of the hepatic artery has a gradually increasing galactose content after galactose injection, and the amount of blood entering the liver by this vessel is only about one-fifth to one-sixth of the total blood supply of the liver.

In the data of table 3 a hyperglycemia following galactose injection is shown. In the control experiments with rabbits 6 and 7, in which physiological saline solution was injected instead of galactose, a hyperglycemia occurred also, but of a very mild degree as compared with the increases in blood glucose which occurred following galactose injection. It is obvious from these experiments, as well as from those described in the first part of this paper, that glucose is formed in the rabbit following galactose administration.

The data of table 3 are of considerable importance in showing that the increased production of glucose following galactose administration takes place in the liver. The control pairs of blood samples collected before galactose injection show an increase in the glucose of the blood of the hepatic vein. This increase represents the glucose-forming capacity of the liver under the conditions of anesthesia and surgical procedure of the experiment, and ranged from 16 to 31 mgm. per 100 cc. of blood in the 7 experiments carried out. Following galactose injection the increases in the glucose content of the hepatic blood in most instances were considerably greater than those observed in the control pairs collected before galactose administration. This was notably true in the experiments with rabbits 1 and 2 in which the increases in the glucose content of the hepatic blood were 5 and 6 times as great as those observed in the control blood samples.

In the experiments of table 3 it was thus observed that, following galactose administration, there is a decrease in the galactose concentration and a simultaneous increase in the glucose content of the blood which passes through the liver. The conclusion therefore seems warranted that a transformation of galactose into glucose takes place in the liver.

Discussion. In an investigation of the effect of the ingestion of galactose upon the respiratory quotient of dogs, Roe, Gilman and Cowgill (12) found that small doses of galactose did not raise significantly the respiratory quotient of fasted dogs, and that even large doses of galactose did not bring about an appreciable elevation of the respiratory quotient until the second hour after ingestion. These authors interpreted their results as indicating that galactose is not oxidized as such in the dog and that the delayed rise in the respiratory quotient following the ingestion of large doses of galactose was due to an increased oxidation of glucose, a plethora

of which was made available to the tissues by the transformation of galactose into glucose. The data of this report offer supporting evidence for the latter conclusion in two respects. In the first place, the data of table 2, showing no significant withdrawals of galactose from the blood which passes through the leg and the brain of the dog, is confirmatory of the hypothesis of non-oxidation of galactose as such. In the second place, the data of tables 1 and 3 show that, following galactose administration to rabbits, galactose is transformed into glucose and a plethora of glucose is made available in the tissues of this animal, thus establishing a condition which would readily bring about an elevation of the respiratory quotient. The evidence of these two reports thus seems to indicate that in the dog galactose is not oxidized as such, and that for its metabolic utilization it must be transformed into glucose by the liver.

SUMMARY

1. Data have been obtained showing that galactose is transformed into glucose in non-fasted adult rabbits after intraperitoneal administration.

2. Following the administration of galactose, analyses of afferent and efferent blood to the leg and the brain of the adult dog showed arterial-venous differences in galactose concentration that are essentially within the limits of experimental error.

3. Analyses of simultaneously collected samples of portal and hepatic blood of rabbits, following the injection of galactose into the duodenum, showed significant withdrawals of galactose from, and corresponding additions of glucose to, the blood which passed through the liver.

4. The data of this report, together with previously published work, suggest that in the adult dog galactose is not oxidized as such and that its normal metabolism is conversion into glucose, a process which takes place in the liver.

Grateful appreciation is expressed to Smith, Kline & French, Inc., of Philadelphia, Pa., for contributing the galactose used in this research.

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EXPERIMENTAL FEVER IN SYMPATHECTOMIZED ANIMALS

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The importance of the sympathetic division of the autonomic nervous system in the normal regulation of body temperature has been demonstrated by the investigations of Cannon, Newton, Bright, Menkin and Moore (1929), and by those of Sawyer and Schlossberg (1933), but its importance in fever is a matter concerning which there is still considerable controversy.

During the onset of the febrile reaction which follows the injection of bacterial vaccines or foreign proteins there occurs a decrease of the flow of blood to the peripheral tissues of the body (Hewlett, 1911; Stewart, 1911; Fremont-Smith, Morrison and Makepeace, 1929). Cramer (1916, 1924, 1926) has repeatedly emphasized the importance of the sympathetic nervous system in the normal regulation of body temperature, as well as its importance in fever, but apparently he has never investigated fever in sympathectomized animals. Recently I have shown (1934) that in the rabbit the decrease of temperature after the injection of typhoid-paratyphoid vaccine is much more pronounced in the normally innervated than in the sympathectomized ear. Because of this fact it was decided to investigate experimental fever in animals which had undergone destruction of varying amounts of the sympathetic outflow to other regions of the body. The results of this investigation are reported herein.

METHODS. Cats were used exclusively in these studies. Experimental fever was induced by typhoid-paratyphoid vaccine[1] injected intravenously. The amount of vaccine administered varied between 0.25 cc. and 0.50 cc. per kgm. of body weight. Early in the course of these studies it was found that, within fairly wide limits, the intensity of the febrile reaction was quite independent of the quantity of vaccine injected.

All temperature records, both in the normal and the febrile state, were obtained from unanesthetized animals which had been trained to lie quietly on a soft pad during each determination. A clinical thermometer

[1] A vaccine supplied by the Massachusetts Toxin-Antitoxin Laboratories, and containing a billion killed typhoid organisms and a billion and a half killed paratyphoid organisms per cubic centimeter.

was held snugly in the inguinal region, with the animal's thigh pressed firmly against the abdominal wall, for 6 to 7 minutes before taking the reading. At least one hour before injection of the vaccine the cats to be used for an experiment were placed in cages in the room in which the observations were to be made. This was done to allow body temperature to become adjusted to room temperature. One or two determinations were made just before the injection to ascertain the initial temperature. After the injection the determinations were made at intervals of 10 to 20 minutes during the following 3 or 4 hours, and thereafter at intervals of 30 to 60 minutes until the body temperature had started on its return toward normal.

Although no attempt was made to maintain a constant room temperature, it seldom varied more than 2 or 3°[2] during the course of an experiment. There was no consistent relationship between changes of room temperature and the intensity or duration of the febrile response.

In several cats records of febrile responses were obtained while the animals were in the normal state, and again on the same animals after they had recovered from the operations which rendered them completely sympathectomized. This routine was not continued throughout the series of observations, however, because it was found that many of the cats seemed to become somewhat refractory to the vaccine after the first two or three injections. In order to eliminate this as a possible factor in the differences observed between the febrile response in normal and sympathectomized animals, most of the cats were given no vaccine until they had recovered from the operations.

In so far as possible the experimental animals were kept under uniform conditions, as regarded nutrition and general state of health, during the periods in which they were being studied.

One group of animals employed in these studies had undergone bilateral removal of the sympathetic chains from the stellate ganglia to the fourth or fifth lumbar ganglia inclusive. For convenience they will be referred to as animals of group A (*sympathectomized*). The technique employed in removing the sympathetic chains was that described by Cannon, Newton, Bright, Menkin and Moore (*loc. cit.*). Observations were also made on cats in which there remained only the sympathetic outflow to the splanchnic region; cats with gray rami cut. These will be referred to as group B (*only splanchnic outflow intact*). The operative technique used on these animals was similar to that for complete sympathectomy. Langley (1894) has shown that in the lower thoracic region it is possible to distinguish between the gray and white rami of the sympathetic chain with a fair degree of certainty. This information was utilized in the operations

[2] All temperatures are given in degrees centigrade.

for the removal of the sympathetic outflow to the periphery. The gray rami were isolated and sectioned from about the level of the fourth thoracic vertebra to the diaphragm on both sides, care being taken to spare the white rami. The remainders of the sympathetic chains in the thoracic region were removed in toto; also the chains in the abdominal region. As in the case of complete sympathectomy, the operation was done in two stages; the abdomen and one side of the thorax at the first stage, and the other side of the thorax at the second stage. No temperature studies were made until the animals had recovered completely from the effects of the operation (10 to 15 days). It was possible to determine the success of this operation by noting whether hair became erected on the back as the result of exposure to cold. A two-stage operation was also employed for the animals in which it was desired to remove only the splanchnic outflow; the splanchnic nerves of one side being sectioned at each stage. These animals will be referred to as group C (*no splanchnics*).

Three animals with inactive adrenals were studied in an attempt to differentiate between the effect on the fever curve of merely inactivating the adrenals (leaving intact all, or at least half of the splanchnic outflow) and that of completely excluding the splanchnic innervation. The liberation of adrenine is known to accelerate the oxidative processes of the body (Aub, Bright and Forman, 1922; McIver and Bright, 1924). Furthermore, Cannon and Pereira (1924) have demonstrated that adrenal stimulation occurs in fever. In two cats of the present series the adrenals were inactivated by removal of the right gland and denervation of the left. The denervation was accomplished by section of the left splanchnic nerves and removal of the left abdominal sympathetic chain from the level of the fourth or fifth lumbar vertebra to the diaphragm. In a third animal one adrenal was removed and the other demedullated by means of suction. Since these animals gave fever curves practically identical with those of group C they will be classed as group C_1.

At the conclusion of the experimental studies the animals were sacrificed and autopsies performed in order to verify the record made at the time of operation.

Results. It is known that cats, as well as other warm-blooded animals, show diurnal variations of body temperature. For this reason hourly temperature observations were made on two cats throughout a twenty-four hour period to determine the variations which were to be expected under the conditions of the experiments. It was found that the highest body temperature occurred between 8:00 and 12:00 p.m., and the lowest between 6:00 and 10:00 a.m. The maximal variation over the twenty-four hour period was 1.0°; during the period between 9:00 a.m. and 10:00 p.m., however, the maximal variation was only 0.5°.

The febrile response in normal and sympathectomized cats. In normal

cats the febrile reaction began 20 to 30 minutes after the injection of vaccine, and the maximal temperature increase occurred usually within 3 hours. The fever curves from these animals show two distinct peaks

TABLE 1

Variations of body temperature after the intravenous injection of typhoid-paratyphoid vaccine

EXPERIMENT NUMBER	INITIAL BODY TEMPERATURE	MAXIMAL INCREASE OF BODY TEMPERATURE	TIME AT WHICH MAXIMAL TEMPERATURE OCCURRED	NUMBER OF DISTINCT PEAKS IN FEVER CURVE	EXPERIMENT NUMBER	INITIAL BODY TEMPERATURE	MAXIMAL INCREASE OF BODY TEMPERATURE	TIME AT WHICH MAXIMAL TEMPERATURE OCCURRED	NUMBER OF DISTINCT PEAKS IN FEVER CURVE
Controls					Group A*				
	°C.	°C.	*minutes*			°C.	°C.	*minutes*	
13	39.30	1.90	103	1	40	37.00	2.17	358	0
16	38.60	2.50	50	2	44	38.33	1.22	343	1 (?)
20	39.00	2.06	209	2	46	38.06	1.33	578	1
22	37.66	2.78	221	2	48	37.89	0.77	294	0
26	38.66	1.40	73	1	50	37.50	1.80	474	0
28	38.44	1.11	120	2	52	38.11	1.19	464	0
29	38.83	1.39	245	2	56	38.00	1.39	567	1
33	38.66	1.67	83	2	63	37.93	1.45	497	0
35	38 61	2.94	102	2					
62	38.77	1.39	226	2					
65	38.44	1.72	64	2					
66	38.44	1.56	223	2					
Averages...	38.62	1.87	143			37 85	1.41	447	
Group B†					Group C‡				
15	39.15	0.65	230	1	30	39.22	1.28	49	1
17	38.00	1.60	291	2	32	38.17	0.83	55	2
21	37.72	2.16	250	2	53	37.95	1.99	309	2
23	38.66	1.11	315	2	57	38.61	1.56	227	2 (?)
49	38.44	1.78	434	0	58	38.33	1.55	282	2
54	36.89	2.44	481	0	61	37.82	2.40	290	2
67	37.88	1.56	473	1	64	38.71	1.06	239	2
Averages...	38.10	1.61	353			38.26	1.52	207	

* Completely sympathectomized.

† Peripheral sympathetic outflow abolished; splanchnic outflow intact.

‡ Splanchnic nerves sectioned.

(table 1 and fig. 2)—one within 1 or 2 hours after the injection, and the other within 3 or 4 hours. The first peak was generally higher than the second. The changes of body temperature which gave rise to these peaks were usually rather abrupt in the normal animals. The fever curves for

the group A animals were conspicuous by the absence of distinct peaks. They exhibited a gradual, prolonged rise of temperature, with a maximum 6 to 8 hours after the injection. Representative fever curves from a control animal and from an animal of group A are given in figure 2.

The most outstanding difference between the febrile response in normal animals and in those of group A was the time required for the attainment

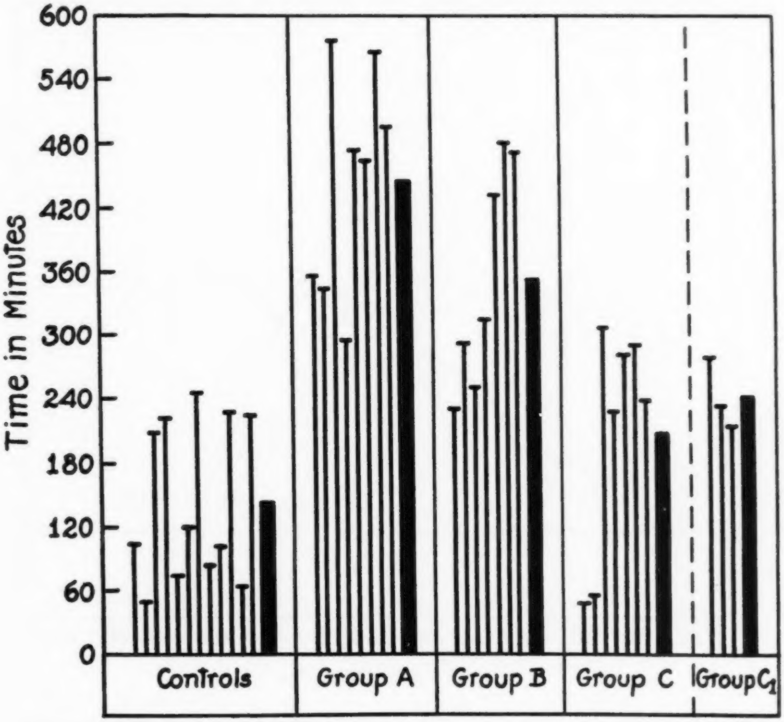

Fig. 1. Time required for attainment of the maximal increase of temperature after intravenous injection of typhoid-paratyphoid vaccine. Averages for each group are indicated by heavy black lines.

of the maximal temperature increase. This is shown graphically in figure 1. In the animals of group A the maximal increase never occurred earlier than 294 minutes, whereas in the controls the maximal increase never occurred *later* than 245 minutes. The average for the controls was 143 minutes; for the cats of group A, 447 minutes.

Vigorous shivering was a characteristic of the febrile reaction in most of the animals, both normal and sympathectomized, but there was no con-

sistent relationship between the time at which shivering occurred and the time of maximal temperature. As a rule the most pronounced shivering was seen during the first two hours after the injection of vaccine. In the animals of group A it was frequently noted that the highest temperature was attained several hours after all detectable shivering had ceased.

Erection of hair and dilatation of the pupils were often observed in nor-

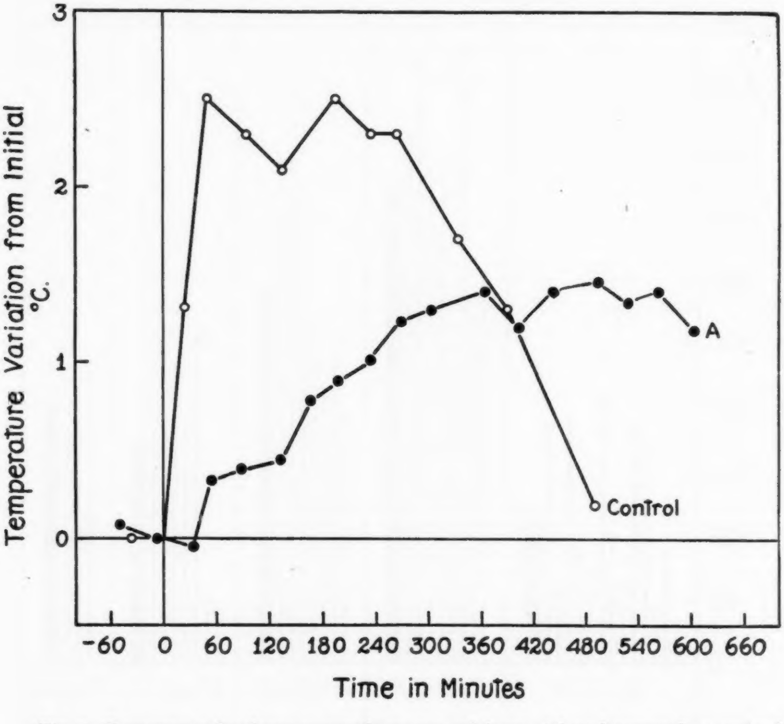

Fig. 2. Representative fever curves from control (expt. 16) and sympathectomized animals (group A, expt. 63). Typhoid-paratyphoid vaccine injected at 0 time.

mal cats during the early stages of the febrile reaction; both phenomena absent in the animals of group A.

In many instances the increase of body temperature was greater in the controls than in the group A cats. The average maximal temperature increase for the former was 1.87° (range 1.11 to 2.94), and for the latter 1.41° (range 0.77 to 2.17) (table 1). It is interesting to note that in the cats of groups A and B the average initial body temperature was somewhat lower than in the controls (table 1).

The effect of destruction of the peripheral sympathetic outflow on the febrile

response. Before making an attempt to explain the delay in the development of fever in sympathectomized cats it was thought advisable to investigate the febrile response in animals in which only the sympathetic outflow to the periphery had been destroyed (cats of group B). With respect to the time required for the attainment of the maximal temperature, these animals were quite similar to those of group A (fig. 1 and table 1). The average time at which the maximal increase occurred in this group was 353 minutes, as compared with 447 minutes for the animals of

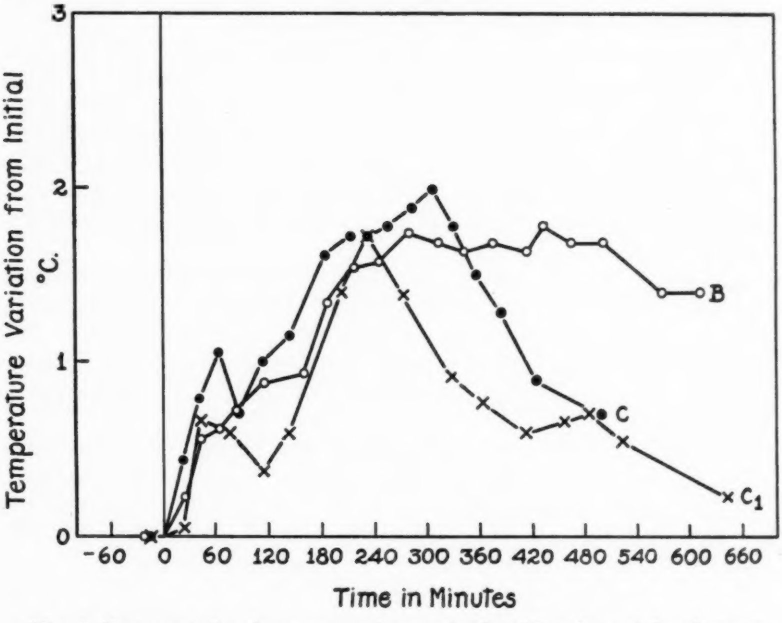

Fig. 3. Representative fever curves from animals with only peripheral sympathetic outflow abolished (group B, expt. 49), only splanchnic nerves sectioned (group C, expt. 53) and only adrenals inactivated (group C_1, expt. 69). Typhoid-paratyphoid vaccine injected at 0 time.

group A, and 143 minutes for the controls. The average temperature increase for group B animals was 1.61° (range 0.65 to 2.44), as compared with 1.41° for group A, and 1.87° for the controls (table 1).

An analysis of the fever curves of animals in group B leads to the conclusion that their febrile response is closely related to that of animals in group A, but the appearance of one or two distinct peaks is more common in the former (table 1). In 3 of 7 experiments there occurred distinct but small peaks within 2 hours after injection, followed by larger peaks 3 to 4

hours later. In no experiment were the peaks so pronounced as in the control animals. A typical fever curve for the animals of group B is given in figure 3. It will be noted that the development of fever in these animals, as in those of group A, was rather slow and gradual.

The effect of splanchnic section on the febrile response. In figure 1 it will be noted that the speed with which the maximal fever was attained in animals of group C approximated fairly closely that for the controls, the average being 207 minutes for the former as compared with 143 for the latter (table 1). The average temperature increase for the cats of group C was 1.56°, as compared with 1.87° for the controls.

A representative fever curve for the animals of group C (fig. 3) shows two fairly discrete peaks. It is interesting to note, however, that the first peak is much smaller in this curve than in the one for the controls (fig. 2). In 5 of 7 fever curves for group C animals there were two peaks. The first was the less prominent in each instance. On the whole, it may be stated that even though the cats in group C gave febrile responses more similar to the normals than did those of groups A and B, the reaction was much more variable.

Although in 3 of the 7 animals in group C there was a definite delay in attaining the maximal temperature increase, the onset of the reaction in each case occurred within a short time after the injection of vaccine. The same was true for the animals of group C_1. This speedy onset of the fever response was also a constant characteristic of the normal cats, but not of those of group A.

The febrile response after inactivation of the adrenals. Since only three animals were studied in this series the results are not recorded in tables 1 and 2; a representative fever curve is given in figure 3. Although the maximal temperature increase appeared somewhat earlier in the group C animals, the general shape of the two curves is almost identical.

For aseptic precautions it was necessary to clip much of the hair from animals which were to undergo surgical operations. In order to eliminate this as a possible factor in the results obtained from operated animals, experiments were carried out on two normal cats from which practically all hair had been clipped. The fever curves from these animals were indistinguishable from those of cats with intact hair covering.

Discussion. The results of the present investigation show that in normal animals the intravenous injection of vaccine results in a febrile reaction which is rapid in its onset, and which produces the maximal temperature increase within one or two hours. Although vigorous shivering was a frequent finding in both the operated and the control animals, it cannot be said that this phenomenon is essential for the development of fever, because in several experiments pronounced febrile temperatures occurred in the absence of any demonstrable shivering. In several other

experiments the interval between the occurrence of shivering and the appearance of the maximal temperature was several hours. Since we cannot rule out the possibility that increase of tension in skeletal muscle caused the elevation of temperature in the absence of shivering, this must be considered in interpreting the results obtained.

Reference has been made to the importance of the sympathetic nervous system in the normal regulation of body temperature. Of especial interest in this connection is the work of Sawyer and Schlossberg (*loc. cit.*), who found that sympathectomized cats were very sensitive to cold, and that they lost heat much more rapidly than normal animals when exposed to cold. On the basis of these observations one would expect the febrile reaction in sympathectomized animals to be abnormal. If we accept the view that fever results from an actual decrease of heat elimination and an increase of heat production, and concede that ablation of the sympathetic outflow destroys the most important mechanism whereby heat loss may be diminished, we are forced to the conclusion that the fever which develops in sympathectomized cats after vaccine injection is the result of greatly increased heat production. The increase must be relatively great, since, in order to elevate body temperature to the fever level, it must compensate for the absence of a mechanism which, if present, would facilitate the process by decreasing heat dissipation.

Aside from the increase of heat resulting from increase of muscular activity in the form of shivering or generalized movement, little is known regarding the mechanisms which may bring about an acceleration of heat production in sympathectomized animals. In spite of differences of opinion regarding the innervation of the thyroid, its involvement in the febrile response after vaccine injection in sympathectomized cats is certainly a possibility worthy of consideration. Although ablation of the sympathetic outflow may remove part, or all, of the nerve supply of this gland, there appears to be no satisfactory reason for assuming that the organ is rendered functionless thereby. Uhlenhuth and Schwartzback (1927), Houssay et al. (1931), and others have demonstrated that the thyroid can be stimulated to activity by a hormone from the pituitary. Neither the pituitary nor the thyroid is essential for the production of fever in animals with an intact sympathetic outflow (Solari, 1931; Borchardt, 1928), but it is possible that in sympathectomized animals these organs assume a more important rôle in this connection.

The slow onset of the febrile reaction and the delayed appearance of the maximal temperature increase in sympathectomized animals are probably caused by two factors; the absence of peripheral vasoconstriction (at least in the early stage of the reaction), and the absence of adrenal secretion. In a previous investigation (Pinkston, *loc. cit.*) it was found that injection of typhoid-paratyphoid vaccine in rabbits always resulted

in rather prompt vasoconstriction in the normally innervated ear. In the sympathectomized ear there was also constriction, but only in about 50 per cent of the cases. In some instances the phenomenon was much delayed, occurring 1 to 3 hours after the injection. Inactivation of the adrenals abolished the early vasoconstriction in sympathectomized ears, but did not appear to alter the delayed constriction.

By removal of the sympathetic nervous system the principal physiological mechanisms for decreasing heat loss are abolished. Not only is the vasoconstrictor innervation destroyed, but also the most important humoral factor that might bring about vasoconstriction. So far as we now know, the only mechanism left intact which could cause vasoconstriction is the pituitary. It is known that extracts of the posterior lobe of the pituitary cause a rise in blood pressure by constriction of the arterioles (Krogh, 1929). Of course there is the possibility of a slight decrease of blood flow to the periphery as the result of a decrease of vasodilator tone, but it is doubtful if this is significant in the development of fever in sympathectomized animals.

That extreme hyperpyrexia can occur in completely sympathectomized cats has recently been shown by Bacq, Brouha and C. Heymans (1934), who employed tetrahydro-β-naphthylamine and α-dinitronaphthol as pyretogenic agents. It is doubtful, however, if the results they secured with these drugs should be compared with those obtained by the injection of bacterial vaccines.

Cats in which only the sympathetic outflow to the periphery has been abolished give fever curves quite similar to those from completely sympathectomized cats. The slight difference which is present is probably due to the fact that in the former preparations the adrenal medulla still retains its innervation. It is questionable, however, whether activity of this structure has any significant influence on peripheral blood vessels with intact sympathetic innervation (Pinkston, *loc. cit.*). After section of both splanchnic nerves there is only a slight alteration in the fever curve. This would seem to indicate that the sympathetic outflow to the splanchnic region is of less consequence in the development of fever than the outflow to the periphery of the body.

The fever curves of cats with adrenals inactivated (group C_1) are very similar to those of animals with splanchnics sectioned. As is true of the latter animals, the time required for attainment of the maximal febrile response is slightly longer, and the first fever peak is decidedly smaller in cats with inactive adrenals than in the normal animals. The fever curve given in figure 3 (C_1) is from an animal in which one adrenal had been removed and the other demedullated. Since this method of adrenal inactivation does not require the section of either of the splanchnic nerves, one is perhaps justified in assuming that the fever curve mentioned is charac-

teristic of animals with inactive adrenals, but with the general splanchnic outflow intact. This indicates that the alterations observed in the fever curves of splanchnectomized cats are to be attributed more specifically to absence of innervation of the adrenal medulla, rather than to ablation of the general splanchnic outflow.

The appearance of two distinct peaks in the fever curves of normal animals has been mentioned above. This matter was discussed by Cannon and Pereira in 1924 (*loc. cit.*). They found in cats that the first rise was rather abrupt, and reached a maximum about 34 minutes after the injection (typhoid vaccine). This rise was accompanied by shivering and erection of hairs; it did not occur after inactivation of the adrenals. The second rise, which developed rather slowly, was not accompanied by shivering. A study of this phenomenon in the experiments of the present investigation has led to conclusions which, in general, confirm those of the above-mentioned investigators. The first temperature rise, or peak, although present in cats with sectioned splanchnics, was much less prominent than in the controls. This finding, plus the appearance of a fairly small but distinct first rise in several of the cats with gray rami sectioned (splanchnics intact), suggests that the early temperature increase characterizing the fever curves of normal animals is the result of two factors; a decrease of heat loss by peripheral vasoconstriction, and an increase of heat production caused by the liberation of adrenine (Cannon, Querido, Britton and Bright, 1927). The fact that either splanchnic section or adrenal inactivation causes less depression of the first fever peak than does section of the gray rami, indicates, however, that this peak is more dependent upon the peripheral sympathetic outflow than upon the splanchnic.

Finally, the relations of the fever curve to the normal and to the different experimental conditions here considered may be briefly stated as follows: the time required for attainment of the maximal temperature progressively *increases* from normal to completely sympathectomized animals (i.e., normal animals $< C < B < A$); and both the temperature level at the first fever peak and the maximal temperature attained *decrease* progressively from normal to completely sympathectomized animals (i.e., normal animals $> C > B > A$).

SUMMARY

1. The intravenous injection of typhoid-paratyphoid vaccine in normal cats results in a febrile reaction which is rapid in its onset, and which produces the maximal increase of body temperature within 1 or 2 hours (see figs. 1 and 2). The fever curves which are obtained show two distinct peaks, one within 1 or 2 hours after the injection, and the other within 3 or 4 hours (fig. 2).

2. Complete removal of the sympathetic chains, from the stellate to the

pelvic ganglia inclusive, causes a pronounced delay, both in the onset of the febrile reaction and in the attainment of the maximal temperature increase after the injection of vaccine (see figs. 1 and 2). In general, fever curves from these animals show no clear-cut peaks (see fig. 2).

3. Cats which have undergone ablation of the sympathetic outflow to the periphery of the body, with the splanchnic outflow intact, yield fever curves quite similar to those from completely sympathectomized cats, except for the persistence of a slight first peak in the former (see fig. 3).

4. Fever curves of cats with the splanchnic nerves sectioned, or with adrenal inactivation, are only slightly different from those of normal cats. This difference consists of a small delay in the onset of the reaction and in the attainment of the maximal temperature increase; also the first peak of the fever curve is smaller than in the normal animals (see fig. 3).

5. As a general rule the injection of vaccine causes a greater increase of body temperature in normal than in operated cats. The average maximal temperature increase for the controls was 1.87°; for the completely sympathectomized cats, 1.41°; for the cats with only the peripheral outflow destroyed, 1.61°; and for those with only the splanchnic outflow abolished, 1.56° (see table 1).

6. Fever can develop in sympathectomized cats in the absence of detectable shivering.

I wish to express my sincere gratitude to Prof. W. B. Cannon for suggesting this problem, and for his advice and encouragement during the investigation.

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DISTRIBUTION OF GLUCOSE IN BLOOD

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There have been several recent attempts to determine the distribution of sugar between plasma and corpuscles in blood (Folin and Svedberg, 1930; MacKay, 1932; Somogyi, 1933, etc.). In these investigations an anticoagulant, oxalate, or heparin, was added. Such a procedure entirely disregards the possibility pointed out by Irving and Kay (1926) that anticoagulants may cause a change in the permeability of the corpuscles, and in consequence may profoundly influence the values obtained. If it be true that an anticoagulant renders the corpuscles more permeable to sugar, then the quotient $\frac{\text{corpuscle sugar}}{\text{plasma sugar}}$ found for oxalated or heparinized blood is merely an indication of the extent of damage done to the corpuscles by the anticoagulant, and does not in the least describe the distribution of sugar in normal circulating blood.

It is not necessary to follow Irving and Kay's method of centrifuging a dissected vein containing blood in order to separate plasma and corpuscles without using an anticoagulant. Significant results are obtained if the blood is drawn under oil with sufficient care and speed, and if it is centrifuged immediately at high speed (2000 revolutions per second) under oil for 2 minutes.

In rabbits, guinea pigs, rats and cats the blood for these experiments was drawn directly from the heart; in dogs, either from the heart or the femoral vein; in man, from an arm vein.

The procedure was as follows: 15 to 30 cc. of blood were drawn through a fairly large needle into a syringe containing about 2 cc. of oil. Approximately 6 cc. were ejected under oil into a centrifuge tube and centrifuged at once; 1 cc. of the plasma thus obtained was used for determination of total reducing substances by the Folin-Wu method, and 1 cc. for glucose by Somogyi's Zn or Cu method. In the meantime, another 3 cc. of blood was ejected from the syringe into a test tube under oil, and 1 cc. samples were immediately withdrawn for determination of the total reducing substances and glucose of whole blood. This whole procedure took less than 4 minutes, and all samples were discarded if there was any evidence of

clotting. The remaining blood was ejected into a tube containing the appropriate amount of oxalate. The oxalated blood was used for a hematocrit reading (it was found that the same reading was obtained on oxalated and unoxalated blood), and for determination of total reducing substances and glucose, both of whole blood and plasma.

The proportion of glucose and non-glucose reducing substances in plasma and corpuscles was calculated for 100 cc. of whole blood as follows:

(1) Plasma glucose = per cent plasma (obtained from hematocrit reading) × value obtained from plasma treated with Somogyi's reagent.

TABLE 1

Distribution of reducing substances in blood

ANIMAL	WITHOUT ANTICOAGULANT				OXALATED			
	Plasma glucose	Plasma non-glucose	Corpuscle glucose	Corpuscle non-glucose	Plasma glucose	Plasma non-glucose	Corpuscle glucose	Corpuscle non-glucose
Rabbit	105	0	0	20	83	8	20	14
Rabbit	83	0	0	28	73	3	23	13
Rabbit (before insulin)	134	1	0	26	126	9	12	14
Rabbit (after insulin)	23	9	0	17	23	6	0	20
Rabbit (after adrenalin)	322	3	0	20	280	7	35	18
Rat	109	7	0	19	95	8	26	8
Rat (light ether anesthesia)	127	5	10	20	110	12	20	11
Rat (ether anesthesia)	97	17	12	9	85	12	36	2
Guinea pig	108	1	0	29	102	10	12	14
Guinea pig	117	3	0	22	107	7	15	18
Dog (before amytal)	90	2	3	23	88	4	5	21
Dog (after amytal)	91	0	2	25	89	0	3	25
Dog (under amytal)	106	0	2	12	100	2	8	10
Dog (after ether)	356	5	8	19	321	4	47	12
Cat (under amytal)	87	3	0	11	72	9	16	5
Cat (under amytal)	100	0	5	15	94	6	14	6
Cat (after adrenalin)	360	14	4	22	315	7	53	25
Man	78	3	11	19	67	4	24	16
Woman	76	2	11	22	72	3	19	24
Man (after meal)	108	1	10	24	95	9	30	10
Man (blood chilled)	77	2	5	16	56	5	33	25

(2) Plasma non-glucose reducing fraction = (per cent plasma × value from plasma treated with Folin-Wu reagent) − plasma glucose (1).

(3) Corpuscle glucose = value from whole blood treated with Somogyi's reagent − plasma glucose (1).

(4) Corpuscle non-glucose reducing fraction = value from whole blood

treated with Folin-Wu reagent − [plasma glucose (1) + plasma non-glucose (2) + corpuscle glucose (3)].

Typical results are given in the accompanying table. In all the experimental animals except man there is clear-cut distribution of glucose and non-glucose reducing substances, the former being present exclusively in the plasma, the latter in the corpuscles. The addition of oxalate in every case caused a redistribution of glucose, the corpuscles taking it on and the plasma losing it. Amytal anesthesia in the cat and dog does not appear to affect the permeability of the corpuscles to glucose. A short period of light ether anesthesia produced a very slight increase in corpuscle sugar in the dog and rat. Adrenalin in physiological doses does not affect the permeability of the corpuscles, and even with very high "blood sugar," e.g., over 300 mgm. per cent, the corpuscles contain no glucose. Insulin and low "blood sugar" likewise do not cause a redistribution of glucose.

In man the permeability of the corpuscles appears to be more easily disturbed than in the other animals studied. If no precautions other than those outlined above are taken, the corpuscles are found to contain 10 to 16 mgm. per 100 cc. of blood. If the syringe and containers with their oil are all thoroughly chilled by keeping them in cracked ice throughout the experiment, the corpuscle glucose now approaches very nearly the limit of experimental error. Further evidence of the less resistant nature of the human corpuscle is shown by the fact that addition of oxalate to human blood causes a greater amount of glucose to pass into the corpuscles than under the same experimental conditions in the case of the rabbit, etc.

CONCLUSION

Using proper precautions, the corpuscles of the blood of the rabbit, rat, guinea pig, cat, dog and man are found to contain no glucose. This is true whether the "blood sugar" is high, as after adrenalin, or low, as after insulin. Addition of oxalate renders the corpuscles permeable to glucose, those of man being more susceptible than the other mammals studied.

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ON THE RELATION BETWEEN THE HEART RATE DURING EXERCISE AND THAT OF THE IMMEDIATE POST-EXERCISE PERIOD

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The available data concerning the pulse rate during exercise are very scanty. Until the advent of the cardiotachometer it was practically impossible to obtain these data in the most strenuous exercise since the movements of the subject interfered so profoundly with the records obtainable by all previous methods of registration.

Although Boas and his associates have published a considerable amount of data concerning heart rate during exercise no attempt has been made to study the change that occurs during the abrupt transition from active exercise to rest. Although further, many data exist concerning the heart rate after exercise, the importance attaching to that during the work is naturally greater.

A logical outcome of the foregoing is therefore to examine the possibility of predicting the heart rate during exercise from observations made immediately after exercise.

During the course of an investigation of the heart rate during and after exercise, a considerable number of records was taken by means of a modified Boas cardiotachometer. An examination of these records was made with the above purpose in view.

METHOD. The cardiotachometer record was written as an ink tracing on a fairly rapidly moving narrow strip of paper, the time being inscribed each second. The exercise in all cases was carried out on a flat treadmill surface travelling at various speeds from about four and a half miles to about eleven miles per hour, but with uniform speed in any given experiment. The duration of the exercise was very variable, being adjusted to the intensity of work, so that for the most part, except where the subject was running very fast, he continued long enough to attain a fairly steady state. In the case of the slower speeds the treadmill surface was inclined upwards so as to increase the intensity of the exercise. The subjects were normal men in the third or fourth decade. Our conclusions reached from study of such subjects may not apply to boys and certainly do not apply to dogs.

On examining the records for the purpose in hand, the heart rate was

determined for four periods of ten seconds each, two immediately preceding the stop signal and two immediately following this signal. To secure an accurate result it was necessary to count the beat intervals and fractions thereof to the nearest 0.1 in each ten second period. The result multiplied by 6 (approximated to the nearest whole number) gave the heart rate per minute correct to one beat (excluding errors of timing).

TABLE 1

Heart rate during consecutive ten second periods

(1 and 2 immediately before and 3 and 4 immediately after cessation of exercise)

NO. OF SUBJECT	1	2	3	4
1	136	136	132	123
1	141	142	140	135
2	160	160	166	154
2	180	181	186	174
3	164	176	172	168
4	186	183	178	168
4	194	192	188	181
4	182	184	186	175
5	104	105	98	89
5	146	141	141	122
5	166	166	162	154
6	142	142	142	133
6	156	157	154	142
6	164	174	172	160
1	160	161	159	153
1	163	164	161	157
Mean............	159.0	160.2	158.6	149.2
Approximate mean..........	159	160	159	149

TABLE 2

Heart rate during consecutive ten second periods

(1 and 2 immediately before and 3 and 4 immediately after cessation of work)

NO. OF SUBJECT	1 −20	2 −10	3 +10	4 +20
7	176	177	174	154
1	128	128	122	126
8	194	189	185	165
9	176	183	183	166
9	126	136	124	100
9	182	186	186	177
9	194	189	196	187
9	186	190	189	180
9	189	186	186	186
9	180	180	183	179
9	189	186	183	180
10	199	200	202	194
11	108	108	105	105
12	195	200	190	175
11	198	197	194	180
7	185	188	185	180
1	171	168	159	158
7	150	145	144	137
7	160	162	163	156
1	144	144	144	145
7	141	145	146	124
7	103	102	107	95
1	159	159	169	170
Mean............	166.6	166.9	166.0	157.3
Approximate mean..........	167	167	166	157

Errors of draughtsmanship (erecting vertical lines and judging tenths of a beat interval) may be reckoned as negligible. The results are set out in the two tables in the arbitrary order of date, with the object of testing whether stabilised averages were being obtained. The first group includes six subjects observed on different occasions up to June 1930, while the second includes seven subjects from this date up to April 1932. One subject only was common to the two groups.

The mean data show agreement to within less than one heart beat in the two consecutive periods of work. It is also noteworthy that the decrease in the heart rate in the first ten seconds after the cessation of the exercise was almost identical in each case. Moreover the fact that in both cases the mean values for the two periods during the exercise agree to the nearest whole beat is evidence of the stability of the mean results.

From this it is clear that the heart rate falls very little during the first ten seconds after the work, and for the next ten seconds only about 6 per cent.

The value of the result for prediction purposes in individual cases must, however, rest on the extent of individual variation in these data. To investigate this point these variations were collected from both tables, namely: the individual differences in heart rate in the two consecutive periods of exercise (from columns 1 and 2) and the individual differences in heart rate that takes place immediately on cessation of work (from columns 3 and 4).

An analysis shows that the S.D. of the variation in heart rate during the exercise periods is 4.03, i.e., about 2.6 per cent, while the S.D. of the change in heart rate in the first ten second period after the exercise is 4.58, i.e., about 2.9 per cent.

Merely for the sake of completeness a slight correction[1] should be applied to the latter figure bringing it to 2.8 per cent, since the difference of the two mean heart rates in question is about one beat.

It may therefore be deduced from the foregoing that the pulse rate during exercise may be predicted from that recorded immediately after exercise[2] with an error whose S.D. is rather less than 3 per cent.

SUMMARY

An examination of heart rate data recorded by means of a cardiotachometer in the case of strenuous exercise on a treadmill shows that in the ten seconds following cessation of the exercise the heart rate of man decreases on an average of just about one beat per minute. Analysis of the data indicates the possibility of predicting the heart rate during exercise from that obtaining in the ten second period following its cessation with a reasonable degree of accuracy (mean error less than three per cent).

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[1] This correction is applied according to the formula (True S. D.)^2 = (Uncorrected S. D.)^2 + x^2, where x is the difference between the two means in question.

[2] It should be emphasized that "immediately" implies the 10-second period following cessation of work. As may be seen in the tables, there is apt to be a rapid decrease in rate in the second 10-second period. The decrease may be even more precipitous in later periods.

CHANGES IN HUMAN CEREBRAL BLOOD FLOW CONSEQUENT ON ALTERATIONS IN BLOOD GASES[1]

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Although the cerebral circulation of man is of the utmost interest and importance, observations in this field have been fragmentary or indirect. The following procedures have been used: inspection of the vessels of the retina, or of the meninges at operation, measurements of changes in the pressure of the spinal fluid or of the arterial blood, and measurements of the alterations in the gases of blood drawn from an internal jugular vein. An instrument has recently been developed by one of us (1) which makes it possible to obtain a running record of the changes occurring in the flow of blood through an internal jugular vein of unanesthetized human subjects. By means of this device, we have completed observations of the cerebral blood flow in human subjects with respect to seizures (2), syncope (3) and sleep (4), the injection of ergotamine (5), and of various drugs known to influence circulation (6). In the present communication, we report the effect of changes in the composition of respired air.

Material and method. Briefly, the thermo-electric blood flow recorder which was used consists of a stilet fine enough to be introduced through the lumen of a 19 gauge hollow needle into the lumen of a blood vessel so that the tip of the stilet projects into the blood stream. The tip is heated by means of a constant electrical current to a temperature a little higher than that of the blood. If the blood flows faster past the tip, the tip becomes cooler; if the blood flows more slowly, the tip becomes warmer. The temperature of the tip is measured by means of thermojunctions in series with a galvanometer. In order that changes in body temperature should not be misinterpreted as a change in flow, the cold junction is mounted on the stilet behind the hot junction; it then becomes possible to measure not the absolute temperature of the tip, but the difference in temperature between the heated tip and the body of the stilet. This difference in temperature varies only slightly with changes in body temperature but greatly with changes in blood flow.

[1] This study was aided by a grant from the Josiah Macy, Jr. Foundation. This is paper no. 38 in a series from the Department of Neurology, Harvard Medical School, entitled "The Cerebral Circulation."

The instrument records changes in the velocity of the surrounding stream. If, however, the cross section area of the stream remains constant, changes in velocity may be interpreted as changes in volume flow. We punctured the internal jugular vein close to its point of exit from the skull. Where the vessel pierces the skull, it is surrounded by a ring of bone. We believe that in this part of the vessel sudden changes in calibre would not occur and that the changes in velocity recorded by our instrument were due to changes in volume flow.

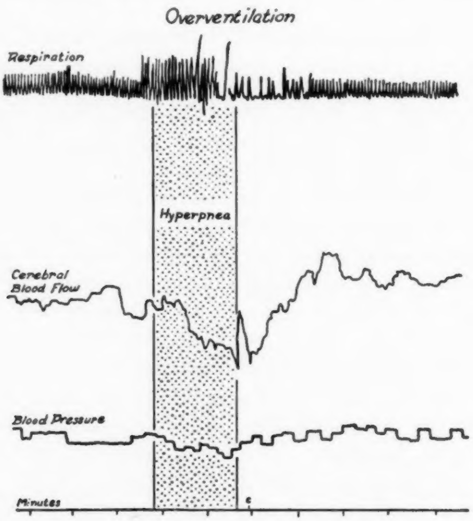

Fig. 1. Effect on cerebral blood flow of voluntary hyperpnea.

The tracings from above downward are: the respiratory movements; the blood flow through an internal jugular vein; the systolic blood pressure (taken by auscultatory method) and, at the bottom, the time recorded in minutes.

During the period marked overventilation, the subject breathed deeply and at an increased rate. At *C* he coughed.

The data obtained are only roughly quantitative. They furnish no absolute values for volume flow; they indicate only the direction and general magnitude of alterations in volume flow. Because it is impossible to duplicate exactly the position of the stilet in the vein, records from separate experiments are only roughly comparable as regards the magnitude of the changes. They are, however, strictly comparable as regards the direction of these changes. Respiratory movements and systolic blood pressures were recorded simultaneously with cerebral blood flow. Our subjects were patients, mostly epileptics who were in the hospital for careful study.

RESULTS. *Decreased carbon dioxide.* In 13 instances the patient was asked to breathe deeply and exhale forcibly for several minutes. In 10 instances this procedure resulted in sudden and pronounced decrease in the flow of blood through the internal jugular vein. In three instances little or no change was observed.

The abruptness and the extent of the decline in flow varied greatly in individuals. One of the responses is shown in figure 1. In this instance the decrease in flow began about 30 seconds after hyperpnea began and persisted until normal breathing was resumed, after which it rose to the normal level. In several cases a fall in blood pressure occurred during the

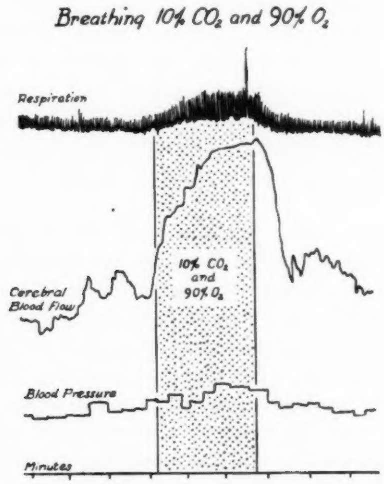

Fig. 2. Effect on cerebral blood flow of breathing a mixture rich in CO_2 and O_2. The tracings are the same as in figure 1.

During period marked 10 per cent CO_2 and 90 per cent O_2, subject breathed this gas mixture through a face mask.

hyperpnea, but the decrease in flow appeared at many points to be independent of the change in pressure and also was of greater magnitude than would be expected from the observed change in pressure alone.

Increased carbon dioxide. In order to increase the CO_2 content of the blood, subjects respired (by means of a face mask and bag) from a tank containing a gas mixture of 10 per cent CO_2 and 90 per cent O_2, the standard mixture for resuscitation used in this hospital. We knew from previous work (7), supported by experience with pure oxygen mentioned later, that the high percentage of oxygen in this mixture did not appreciably affect cerebral blood flow.

Thirteen of these experiments were carried out. In nine instances, there was a prompt increase in blood flow which was maintained as long as the inhalation was continued. In one instance there was no change in flow and in three there was a decrease. In one of these last cases, though a decrease in flow occurred during the breathing, at its close there was a sharp increase. Measurements were not made of the gaseous content of arterial blood during the period of inhalation, so we are not sure in all these instances that the CO_2 content of the arterial blood was actually increased above its usual level. It is possible that in those cases in which flow did not increase there was leakage of air about mask or valve, and an increase in alveolar CO_2 was not effected.

In several instances, an increase in blood pressure occurred while the CO_2 mixture was being breathed, but the increase in flow did not faithfully follow the increase in pressure and the increase in flow was of greater magnitude than would have been expected had it been produced simply by the rise in pressure.

One of the records is reproduced in figure 2. In this, as in most cases, the increased flow persisted for a short time after the breathing of room air was resumed.

Discussion of CO_2 effects. We find that "blowing off" CO_2 from the alveoli of the lungs and from the blood caused a decrease in cerebral blood flow, whereas breathing a high concentration of CO_2 caused an increase. The interesting question arises concerning the mechanism responsible for these changes in flow. Alterations in systemic arterial blood pressure may play a part, but lack of any close correlation of blood flow and blood pressure curves requires another explanation. The explanation lies, we believe, in alterations in calibre of the cerebral blood vessels in response to changes in the concentration of the blood gases. Increase of the blood CO_2 results in a dilatation and decrease results in a constriction of the arterioles of the brain.

This effect of CO_2 on cerebral blood vessels was indicated by the data previously obtained by two of us on the blood gases of arterial blood in contrast with blood drawn from an internal jugular vein (7). We found that when subjects breathed air rich in CO_2, the A-V difference in the oxygen content of blood passing through the brain was decreased (venous blood became more arterial-like); whereas when CO_2 was "blown off," the A-V difference for such blood increased (venous blood became more venous). These changes of circulation in the brain were oftentimes the opposite of the circulatory changes in the leg. Likewise, Wolff and Lennox (8) observed through a window inserted in the skulls of cats that there was dilatation of cerebral arteries in spinal fluid pressure when CO_2 was added to the inspired air: when animals were made to overventilate a decrease in the calibre of the cerebral vessels and a fall in spinal fluid pres-

sure occurred. These changes could not be accounted for by the changes which occurred in systemic blood pressure. Later Cobb and Fremont-Smith (9) noted a rise in spinal fluid pressure and an increased redness of the retina when patients breathed a mixture of 10 per cent CO_2 and 90 per cent O_2.

Recently Schmidt and his co-workers, using a blood flow recorder, which operates on a principle similar to that employed by us, have investigated the effect of CO_2 on the circulation of the medulla (10) and of the hyperthalamus (11) in cats. Their observations indicate that CO_2 is a powerful vasodilator for the vessels of these regions.

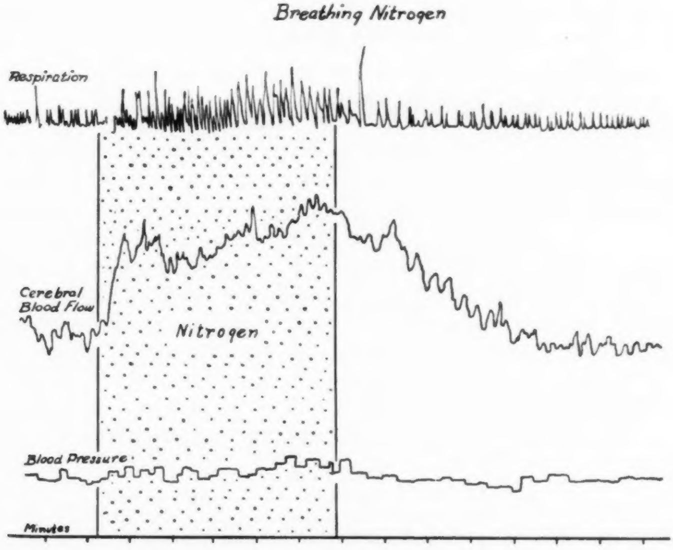

Fig. 3. Effect on cerebral blood flow of a progressive asphyxia.
Tracings are the same as in figure 1.
During period marked nitrogen, the subject breathed pure nitrogen through a face mask.

The evidence from various methods and various investigators, therefore, is in full agreement; an increase in the CO_2 content of the arterial blood causes dilatation of cerebral arterioles and an increase in cerebral blood flow: whereas a decrease in arterial CO_2 produces the opposite effect.

Anoxemia. In six instances an acute anoxemia was induced by having the subject either breathe nitrogen, by means of a face mask and bag, or else rebreathe room air from a small spirometer equipped with a CO_2

absorber. In four instances there was a distinct although, compared to CO_2, a small increase in cerebral blood flow during the inhalation period. As soon as apparent unconsciousness was reached, the breathing of room air was resumed, and the blood flow returned to its former level. No distinctive change in flow occurred in association with either the losing or the regaining of consciousness.

A record of one of these experiments is shown in figure 3.

Breathing oxygen. In four instances, patients breathed oxygen through a mask. The results were equivocal. In three cases there was a slight decrease, and in one a slight increase in flow. The most pronounced decrease is shown in figure 4.

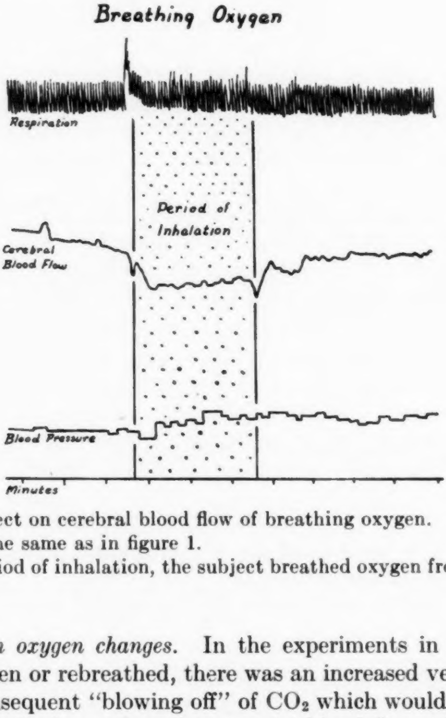

Fig. 4. The effect on cerebral blood flow of breathing oxygen.

Tracings are the same as in figure 1.

During the period of inhalation, the subject breathed oxygen from a bag through a face mask.

Discussion on oxygen changes. In the experiments in which subjects breathed nitrogen or rebreathed, there was an increased ventilation of the lungs and a consequent "blowing off" of CO_2 which would have tended to constrict the cerebral vessels and to restrict blood flow. However, in all but one instance, this constriction effect was overcome, and vasodilatation and increased flow occurred. It seems probable that this cerebral vasodilatation was produced by the decreased oxygen tension of the arterial blood.

Any great change with breathing of pure oxygen is not to be expected

because when room air is breathed, the arterial blood is already almost fully saturated with oxygen. There was in fact no very significant alteration in blood flow, when pure oxygen was breathed. These data are in accord with the observations of two of us on blood gases (7). We found that breathing pure oxygen tended slightly to increase and anoxemia to decrease the A-V difference in the oxygen content of blood passing through the brain. Wolff and Lennox (8) found that anoxemia caused a slight dilatation and oxygen breathing a slight constriction of the pial vessels in cats. Schmidt and his co-workers (10) (11) have also obtained evidence that high oxygen tension constricts and low oxygen tension dilates the vessels of the medulla and of the hypothalamus in the cat. All these observers and methods are in agreement that cerebral circulatory changes consequent on changes in oxygen tensions of arterial blood are small as compared with those due to changes in the tension of carbon dioxide.

CONCLUSIONS

By means of a thermoelectric blood flow recorder, observations have been made of the change in blood flow through an internal jugular vein of unanesthetized human subjects, consequent on changes in the carbon dioxide and the oxygen tension of the blood.

Increase in the carbon dioxide and decrease in the oxygen tension of the arterial blood result in an increase in the cerebral blood flow, chiefly because of the dilatation of the cerebral vascular bed. Decrease in carbon dioxide tension of the arterial blood results in a decrease in blood flow, chiefly because of a constriction of the cerebral vascular bed.

The effects due to changes in CO_2 tension are much more marked than those due to changes in O_2 tension.

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THE EFFECT OF DIFFERENT SIZES OF BALLOONS INSERTED IN THE GUT AND CHANGES IN PRESSURE WITHIN THEM UPON THE ACTIVITY OF THE SMALL INTESTINE[1]

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For the past ten years the balloon method has been employed extensively for studying the intestine of the non-anesthetized dog. Changes in the general tonus, frequency and amplitude of the rhythmic contractions, rate and frequency of the peristaltic contractions, work done, oxygen consumption, etc., have all received attention by experimenters using this method. None of the experimenters, however, seem to have thought of the possibility that different sizes of balloons and varying pressures within them might influence the results obtained or that the seemingly contradictory results obtained in different laboratories might be due to a difference in apparently minor details of procedure. Legros and Onimus (1869), Bayliss and Starling (1899) and Pal (1900) employed the balloon method but do not give the sizes of the balloons used. Langley and Magnus (1905) used animals anesthetized with an A.C.E. mixture. The pressure within the balloon (15 mm. long and 10 mm. diameter) was usually 8 to 12 cm. of water. In their investigation they were unable to record a constant difference in the tracings with pressures varying from 4 to 35 cm. water.

The use of the balloon method in studying the activity of the intestine in Thiry-Vella loops in unanesthetized dogs was first used extensively by Plant (1921). He employed balloons 100 to 120 mm. in length and 15 mm. in diameter under a pressure of 25 to 35 cm. of water. All later investigations carried out by him and his associates (Plant and Miller, 1926; Yonkman, 1929; Gross and Slaughter, 1931) have apparently been done with this same method.

Dvorak et al. (1931), Wilen and Dragstedt (1931) and Orr and Carlson (1933) make no comment on the possible effect of changes in pressure used within the balloon nor do they give the dimensions of the balloons nor the

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pressures employed. Like the above investigators Gruber and his co-workers (1928, 1929, 1930, 1931) make no note of the pressures used in the balloons in their early papers. In reviewing all of the records of this earlier work we find the maximum water pressure used was 15 cm. and in most instances it lay between 10 and 12 cm. These are the pressures given in their later papers (Gruber, Bryan and Richardson, 1932a, 1932b). In all of the experiments the pressure was fairly constant and throughout an experiment it varied not more than 2 mm. in either direction.

Krueger (1934a, 1934b) and Krueger and Howes (1934) employed balloons varying in length from 65 to 95 mm. and 25 mm. in diameter. The pressure within the balloon varied from 30 to 32 cm. of water. Gruber and Brundage (1935) failing to obtain results similar to those reported by Krueger and Krueger and Howes attributed this to the smaller balloon (30 to 50 mm. long and 20 mm. in diameter) and to the lower pressure (15 cm. water) which they used.

This investigation was undertaken to show whether or not the rate of the rhythmic contractions, the tonus and the rate of peristalsis in the unanesthetized dog's intestine could be markedly influenced by changing either the size of the balloons or the pressure employed within them or both.

Method. The method was the same as that employed by us in a previous communication (Gruber and Brundage, 1935) and requires little further comment. Thiry-Vella loops of both the ileum and jejunum were employed. The balloons varied in length from 15 mm. to 120 mm. and in diameter from 15 mm. to 30 mm. The pressures used within the balloon were 5, 10, 15, 20 and 30 cm. of water, each pressure when used being constant to within ± 2 mm. The animals were prepared as previously described and the experiments were performed after the animals had been denied food for 24 hours. In some instances two balloons were used end to end in the same loop of intestine. However the results from such experiments were disappointing particularly in those cases in which the pressure within the two balloons differed.

Results. *Effect of size of balloon upon the rate of the rhythmical contractions.* In the majority of instances as the size of the balloon was increased there was a corresponding increase in the rate of the rhythmical contractions. With a pressure of 15 cm. of water within the balloon the rate of the rhythmic contractions increased from 13 per minute as recorded with a balloon 20 mm. long to 14.9 when recorded with a balloon 120 mm. long. This difference becomes more striking when higher pressures are employed. With a pressure of 30 cm. of water within the balloon the rates changed from 13 to 17 contractions per minute. With the use of pressures of 15 cm. of water or less the increase in rate with balloons varying in length from 20 to 70 mm. is relatively insignificant.

In our results the average rates increased from 13 to 13.3 contractions per minute.

Figure 1 is a record showing the effect on rhythmic contractions of changing the size of the balloon. In A, a balloon 50 mm. long and 20 mm. in diameter was used the pressure in this case being 30 cm. of water. In B, and C, a balloon 120 mm. long and 30 mm. in diameter was employed. In B the pressure was 15 cm. of water, in C it was 30. In A, the rate of the rhythmic contractions is 14 per minute while in C in which the larger balloon was used, and under the same pressure, the rate is 19 contractions per minute. With a long balloon two segmenting contractions slightly out of phase constricting the balloon might be indicated by two small indentations on the record. These might be counted as single contractions. Obviously such counts are erroneous and are particularly apt to occur with

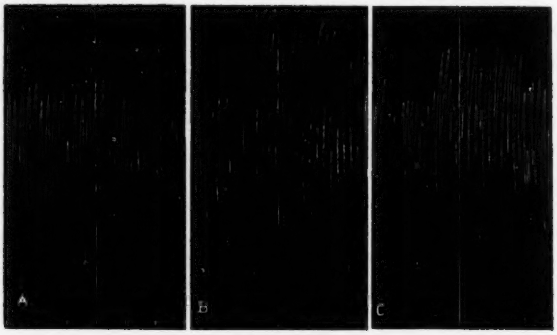

Fig. 1. Top record the rhythmic contractions of the jejunal Thiry-Vella loop Bottom record that of the time in 20 second intervals.

the use of large balloons under high pressures. Generally with the use of small balloons 20 to 50 mm. long the increase in the rate of the rhythmic contractions of the gut induced by increased pressure is lost. This is illustrated in figure 2. In this case an animal with a Thiry-Vella loop of the jejunum was used. In both records a balloon measuring 50 mm. long and 20 mm. in diameter was employed. In 1, the pressure within the balloon was 15 cm. and in 2, 30 cm. of water. The rate in both instances is 12.8 contractions per minute which is about the average rate for the jejunum, 13.3.

Effect of increased pressure within the balloon upon the rate of rhythmic contractions. With the use of long balloons, 70 to 120 mm., increasing the pressure within them usually increases the number of indentations or small contractions in the record. This increase we are inclined to attribute to a second rhythmic contraction influencing the record of the

first. We do not believe this is an actual increase in rate of contraction in a single area of the gut.

In figure 1, B and C, a balloon having a length of 120 mm. and a diameter of 30 mm. was used in a jejunal loop of intestine. In B, 15 cm. water pressure was used, and in C, 30 cm. The average rate in B was approximately 15 contractions per minute, but in C the rate had increased to 19.5 contractions per minute, or approximately 4.5 contractions. What seems to be an increase we believe is actually a second segmenting ring, compressing the balloon while the first is still in force.

Height of rhythmic contractions. With small balloons the height and force of the rhythmic contractions are increased as the pressure is increased within the balloon. This can be seen in figure 2. The balloon was 50

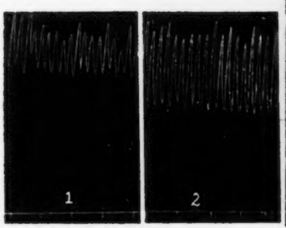

Fig. 2

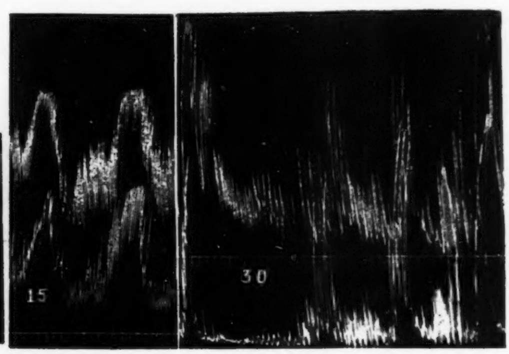

Fig. 3

Fig. 2. Same as figure 1.

Fig. 3. Two balloons were used in this experiment, each balloon being 35 mm. long and 20 mm. in diameter. The time interval is in 20 seconds. The relationship of the levers to the time marker was not disturbed.

mm. long and 20 mm. in diameter. In 2, the pressure was twice that employed in 1 and the height of the contractions is doubled. Although the same result may also occur when large balloons are used, this is usually not the case. In figure 3 a decrease in amplitude is noted with a medium sized balloon (70 mm. long and 20 mm. in diameter) and high pressure. This would seem to be due principally to the failure of one contraction to be completely recorded before another has begun to compress the balloon or to the fact that since the balloon is so long only a part lies within a contraction ring while the rest is in a relaxed portion of the gut. The contraction thus, instead of forcing the water only into the bottle attached to the recording apparatus, forces some of the water into the part of the balloon lying within the relaxed portion of the gut. In this way only a small

contraction is reported where a large one has actually occurred. In figure 1, we note that when the pressure is increased from 15 cm. at B to 30 cm. at C the amplitude of the rhythmic contractions decreases.

Changes in peristalsis with changes in pressure. In some animals increasing the pressure within the balloon induced peristaltic contractions which persisted in some cases but in others disappeared. In figure 3 peristaltic contractions are seen in both records. Upon doubling the pressure within the balloon the number of peristaltic contractions decreased from approximately one every two and a half minutes to one every three and a half minutes. In the animal from which figure 4 was taken 30 cm. water pressure within the balloon always caused, after a temporary stimulation, a complete cessation of peristalsis. With a pressure of 10 and 15 cm. of water, as seen in the record, small peristaltic contractions were always discernible. When the pressure was increased to 30 cm. water the contractions became temporarily more vigorous, then gradually decreased and

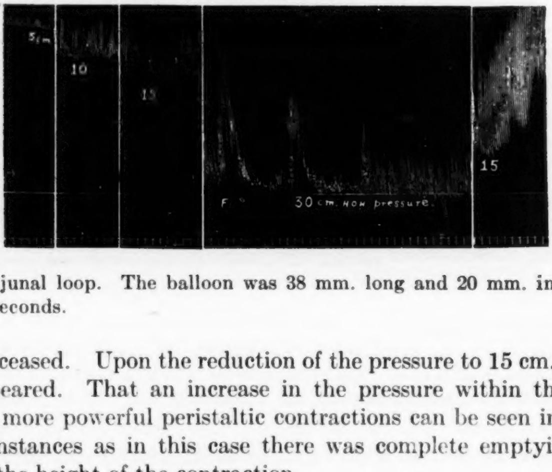

Fig. 4. Jejunal loop. The balloon was 38 mm. long and 20 mm. in diameter. Time in 20 seconds.

ultimately ceased. Upon the reduction of the pressure to 15 cm. of water, they reappeared. That an increase in the pressure within the balloon stimulated more powerful peristaltic contractions can be seen in figure 3. In many instances as in this case there was complete emptying of the balloon at the height of the contraction.

Tonus changes with varying pressures within the balloon. The increase in the height of the rhythmical contractions and of the peristaltic contractions with increases in pressure within the balloon, are mainly due to loss of tonus, and distention of the gut. This distention of the gut can be noted in figure 3 but is best seen in figure 4. In figure 3 the relationship between the recording levers and the time markers was not disturbed. There was a marked decrease in the general tonus, or rather, there was a forced decrease in the general tonus due to the distention of the gut by the higher pressure employed. In figure 4, the height to which the lever would rise were the balloon completely empty was some distance above

the record shown in 5; F marks the point at which the balloon was completely filled supporting a column of water 30 cm. high when held outside the body at the level of the gut. From this record it will be seen that when 30 cm. water pressure was used the balloon walls were actually supporting the column of water during complete relaxation. If the lumen of the gut is greater than the diameter of the balloon when filled, relaxation could not possibly be recorded. If as Plant and Miller (1926) and Yonkman (1929) say the balloon they used just filled the gut but exerted no distending force on account of their "fairly heavy walls" it is apparent that any relaxation or loss of tonus produced by the administration of a drug could not be recorded. It would be possible to record contraction only. On the other hand if the balloon has a large diameter and the gut has to be stretched to accommodate it the organ is then subjected to a continued "distending force." Further relaxation could not be expected to occur under such conditions. Contraction only, therefore, could be recorded.

It is apparent that, in order to confirm or disprove previous work done with the balloon method it is necessary to employ as nearly as possible the same procedure, as to size of the balloon, the pressure within it, a similar section of the small intestine, and the same size dog. Though we have found it not always true, usually a small dog has a smaller lumen of intestine and in such an animal a smaller balloon could consequently be used.

SUMMARY AND CONCLUSIONS

1. The use of long balloons leads to faulty counts of the rhythmic contractions of the intestine due to two segmenting areas influencing the balloon. With balloons 120 mm. in length the rate of the rhythmic contractions is variable, usually 2 to 4 contractions more per minute than when recorded with balloons 20 to 50 mm. in length. This difference is more marked when high pressures (30 cm. water) are used within the balloon.

2. The amplitude of the rhythmic contractions is increased with the increase in pressure within the balloon when short balloons are used. When long balloons (120 mm.) are employed the amplitude may be diminished due to interference, i.e., two segmenting contractions affecting the balloon simultaneously or alternately.

3. In all of these experiments, upon increasing the pressure within the balloon from 15 to 30 cm. of water, the amplitude of the peristaltic waves which had been present under the lower pressure was increased. If they were not already present such an increase in the pressure brought them about. Under continued increased pressure the rate became slower and in some animals the peristaltic contractions ceased altogether.

4. The use of 30 cm. water pressure caused marked distention of the gut and loss of tonus.

5. From our results we believe the pressure within the balloon in Thiry-Vella fistulae of un-anesthetized dogs should never be more than 15 cm. of water (preferably 10 to 12 cm.) In all instances the balloon should be as short as feasible and always of a diameter larger than that of the lumen of the gut. With such balloons and low pressures not only increases in the general tonus can be recorded but also decreases in general tonus as well.

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THE EXPERIMENTAL PRODUCTION IN THE CAT OF A CONDITION SIMULATING PSEUDO-BULBAR PALSY

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Experimental studies of the relation of the cerebral cortex to feeding have been few. It was first found that rhythmic chewing movements could be elicited from circumscribed areas in the cerebral cortex of rabbits. Similar movements were later obtained on stimulation of the cortex of certain insectivores, carnivora, primates, and even of man. Frank (1900) removed symmetrical areas from the motor cortices of monkeys and dogs and thus produced abnormalities of mastication and deglutition. Miller (1920) obtained similar results in rabbits; he was interested in the control of feeding by lower centers after the cortical influences had been removed.

Economo (1902) traced the fibers controlling feeding responses from the cortex down into the internal capsule by the use of the Marchi method. He was able to follow them to the substantia nigra and believed that they ended there. Magoun, Ranson and Fisher (1933) showed by electrical stimulation that the substantia nigra had no control of mastication and deglutition.

Studies of the cortical control of mastication, lapping and deglutition in the cat are difficult due to the fact that the area controlling these functions lies in a region hard to approach. Sherrington (1917) reported that he had been able to elicit rhythmic movements of the jaws and tongue on stimulation of the motor cortex of this animal. Recently Magoun, Ranson and Fisher (1933) have shown by the use of the Horsley-Clarke apparatus that chewing movements occur on stimulation of the most lateral portion of the anterior cruciate gyrus, and rhythmical lapping movements from an adjacent area in the rhinal sulcus. They followed the projection fibers in the internal capsule.

To delimit this cerebral area, it has been projected as closely as possible upon a drawing which we ourselves have used to indicate previous results of stimulation of the cat's cortex. It appears in figure 1 as the region enclosed by a circle. The motor cortex of the cat surrounds the cruciate sulcus. Thus Weed and Langworthy (1926) showed that stimulation of areas A and B anterior to the sulcus (fig. 1) gave rise to movements of the fore-legs while stimulation of areas E and F posterior to the sulcus caused

movements of the hind-legs. In most of the preparations stimulation of area C elicited contraction of the facial and masticatory muscles. No rhythmic movements of the tongue or muscles of mastication were recorded. It will be observed that the region from which these rhythmic movements were obtained by Magoun, Ranson and Fisher lies ventral to the area C of Weed and Langworthy.

METHODS. Twenty-five cats were studied following unilateral and then bilateral cortical injuries, and after death the brain in each case was recovered and examined. The cats were always anesthetized during the aseptic operations. The use of ether was early discontinued because of the extreme excitement that followed the quick recovery from this anesthetic. Nembutal, given intraperitoneally, was substituted due to its marked sedative action. The operation was performed in two stages, only one cerebral cortex being exposed at a time. There was an interval of at least two weeks between the two operations thus allowing the first wound to heal. In many experiments the entire motor cortex and all the cerebral cortex anterior to it was removed in one slice; in other animals more circumscribed areas were cut away.

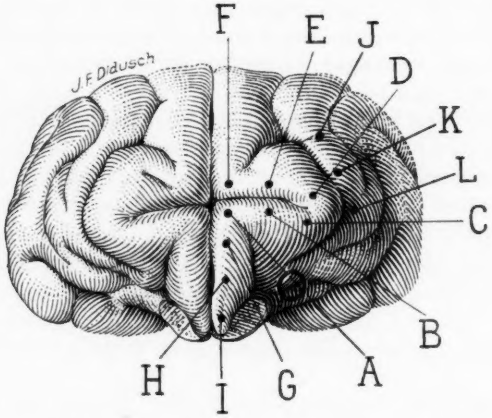

Fig. 1. This drawing shows the electrically responsive and premotor areas of the cat. Bilateral removal of the area surrounded by a circle together with other portions of the cortex gave rise to the abnormalities of eating and drinking, hyperactivity and behavior disturbances described in this paper.

Description of experiments. A constant group of abnormalities was produced by bilateral removal of the electrical responsive motor cortex and all portions of the cortex rostral and ventral to it. The olfactory tracts were sometimes, but by no means always injured. The section was made

so that the lateral ventricle was seldom opened and the caudate nucleus was not exposed.

The cats with the motor cortex removed on one side showed relatively few changes. There was some weakness of the extremities on the side opposite to the lesion which quickly passed away. Disturbances of tone were present in the contralateral legs. The placing reactions which were recently investigated by Bard (1933) were lost in these legs. If a normal cat is blindfolded and the dorsal side of the feet brought in contact with a table, they will be flexed and placed in a standing position upon the table. On the abnormal side this placing did not occur. The legs were not as quickly withdrawn if the foot-pads were touched or pinched as on the normal side, indicating a possible decrease of sensation. The legs on the contralateral side were strongly extended when the cats were held up by head and tail, whereas the unaffected legs were flexed in an effort to escape. The animals drank and ate solid food on the following day, and their methods of eating were those of normal cats.

A radical change in activity followed the operation upon the second cortex. The abnormalities in tone just described were present and bilateral. The fore- and hind-legs were extended on suspending the cats by the head and tail, and the animals were forced by the increased tone to remain immobile. At most there were rhythmic, alternate movements producing flexion and extension of the wrist and claws of the fore-legs. The loss of placing reactions and decrease of sensory response were now bilateral.

Due to changes in tone and sensation, the extremities were held in strange positions. They often were hyperextended in front, behind, or at the side of the body. This was particularly true of the hind-legs which would shoot forward between the fore-legs, or be extended posteriorly. The paws were often so flexed at the wrist and ankle that the cats stood on the dorsal surface of the foot. If the cat was held by the back of the neck with only the hind-legs in contact with the ground, the fore-legs were adducted against the side of the body and flexed at the elbow. The hind-legs were hyperextended and somewhat abducted in walking. If the head was suddenly flexed, or flexed even as in eating, the hind-legs would fly up into the air. On attempting to overcome this difficulty, the hyperextended hind-legs would slide forward past the fore-legs, and the cat fall backwards. When the animal was placed upon its back it lay quietly; the hind-legs were maximally extended, and the fore-legs adducted and flexed at the elbow. The falling reflex revealed a slight unsteadiness.

On the day following the second craniotomy the preparations were able to stand and they walked continually. During the first few days the cats did not turn aside from a straight line to avoid objects. When a corner was reached the animals were unable to proceed. Within two weeks after

the operation they avoided obstacles fairly well. The animals were constantly active for long periods following the second operation; one, in this condition, was kept for three months.

A certain number of animals after either unilateral or bilateral operations tended to move in circles. Following cortical injury upon one side they circled away from the side of the lesion, the normal extremities on the homolateral side were used more efficiently, and forced the cats to circle in the direction away from the sound legs. Those that circled following the second operation walked away from the side of the most recent lesion. This is understood if it is realized that the legs on the side first paralyzed showed considerable recovery, and those involved by the second operation had the greater abnormalities. One animal which had walked in circles away from the side of the second operation was induced on the fifth day to walk toward the side of the lesion in pursuit of food. The legs last involved were so ataxic that the cat lost its balance and fell. Circling, when present, interfered with eating as the animals had to circle correctly in order to reach the plate of food.

The "following reaction" was present in the preparations in which the lesion was complete. The animals would follow any person who was in the room, remaining constantly at their feet. A turn of the investigator would be duplicated by the cat. The animals were eager to be petted; on the other hand they became antagonistic to their former companions, growling and fighting so that it was necessary to house them separately. These reactions were never observed after a unilateral lesion.

After the operation on the second cortex the animals made no effort to eat spontaneously. They would make chewing movements, if a piece of meat was placed in the mouth, and the jaw closed. They would not swallow the food at first, but would extrude it from the mouth. By pushing the meat far back into the pharynx it would eventually be swallowed. Within three or four days the animals would eat without assistance. The cats would snatch at the food greedily until the whole mouth was filled, and then swallow without chewing. Eating initiated emotional reactions usually manifested as loud purring, but at other times they would growl.

Normal cats eat daintily; following the completion of the bilateral operation, the preparations seemed unable to eat unless the fore-paws were in the dish. While eating, the fore-legs showed alternate pawing movements, rhythmical, and of small amplitude, involving the wrist and claws. There was poor coördination between mastication, deglutition and respiration. Swallowing was difficult and required an active extension of the neck. The preparations were no longer particular about their food. The cats almost uniformly would bite on the edges of the dish at some time during the meal. These animals continued to consume an unusual amount of food; they ate ravenously several times the amount required by normal cats.

The difficulties of drinking were more marked than those of eating, and persisted for a longer time. On the first day it was customary to feed the cats from a spoon. Once the milk was introduced into the mouth swallowing occurred. Lapping movements of the tongue often developed; they showed a certain perseveration, and were likely to continue after feeding stopped. After two or three days, the preparations would make unsuccessful attempts to drink from a dish. The animals insisted upon putting their paws into the fluid. Some opened and closed their mouth, and attempted to drink without using the tongue. Lapping became reëstablished only after ten days to two weeks, and even then coördination of lapping, swallowing and breathing was abnormal. Often they would bite on the glass rim, or continue the lapping movements outside of the milk or outside of the dish.

Localization of the lesion. The brain was examined in the twenty-five animals with bilateral lesions to permit a more exact localization of the lesion. The amount of cortex removed was varied in these individuals.

Ten of these cats showed the entire picture of abnormal activity which has just been described. In these preparations the whole electrically responsive area and the cortex anterior to this region had been removed. The olfactory tracts were often injured, but in three cases they were quite intact. We believe that the behavior changes are in no way dependent on a loss of smell. In all of these animals the area from which Magoun, Ranson and Fisher obtained rhythmical lapping and chewing movements was removed on both sides.

In six cats the electrically responsive and the premotor areas were removed bilaterally, but the areas of Magoun, Ranson and Fisher were spared. These preparations showed the abnormalities of tone and sensation which have been described, but their behavior as regards eating and drinking was normal. They were not overactive and did not show the "following response."

The remainder had widespread lesions of the rostral portion of the cortex which did not injure bilaterally the areas circled in figure 1. These animals showed none of the abnormalities of eating, or they appeared to a slight degree and were only transient. Bilateral injury of the cortex including the portions surrounded by a circle in figure one is required to produce abnormalities of eating and drinking, behavior changes, hyperactivity and the "following response."

Discussion. Characteristic abnormalities appear after certain bilateral cerebral lesions in man which are known as pseudo-bulbar palsy. In this condition the motor nuclei of the cranial nerves are not damaged but their supranuclear control is lost. There are difficulties in speaking and eating, and seeming emotional outbursts of laughing and crying. Weakness and hypertonus of the extremities are present in man due to the bilateral

pyramidal tract lesions. Later there is often great improvement. The analogous changes in animals may be summarized under the same headings.

As regards feeding there is difficulty in chewing, swallowing and poor coördination with breathing. The feeding is interrupted by outbursts of growling or more often of purring. The difficulty in drinking is even more profound due to loss of ability to lap, and the incoördination of breathing with drinking. The cats are more friendly with the investigator and more antagonistic with other cats. There is perseveration in lapping, walking and mewing.

The pawing movements which accompany eating, and the persistence in placing the paws in the dish, resemble the behavior of kittens when feeding at the breast. Removal of the cortical areas influencing feeding suggests control at lower levels similar to that of new-born and young kittens.

The increased appetite of the animals is difficult to explain. The suggestion had been given by Fulton and his co-workers (1934) that it is due to increased peristalsis of the gastro-intestinal tract. Pathological hunger may cause the constant walking and mewing as well as their reactions to the investigators and other animals.

These cerebral injuries must interfere with the most elaborate and highly specialized psychic activity. In animals, particularly in the wild state, marked emotional activity is connected with feeding. They must not only capture food, but also protect it from rivals. In man apparent emotional instability accompanies lesions interfering with speaking and eating.

These cortical injuries also produced the tonic abnormalities already described which are not unlike those seen in patients with pseudo-bulbar palsy. The preparations were held by the back of the neck in an erect position with only the hind-feet touching the ground. In this position the fore-legs were adducted and flexed at the elbow so that their position almost duplicated that of the arm in a man with hemiplegia. The fore-legs assumed the same position when the cat was placed upon its back. The flexed arm in man is a result of posture, and is assumed at once by animals when they are placed in the same position.

SUMMARY

Bilateral injury of the rostral portion of the cerebral cortex of cats involving the motor area and particularly an area ventrolateral to the electrically responsive motor area controlling the legs, produces abnormalities of feeding, of behavior, and of tone in the extremities with changes in the righting reflexes. The analogy between certain of these changes and the symptom complex of pseudo-bulbar palsy is discussed. The entire syndrome of pseudo-bulbar palsy would seem dependent on bilateral injury of localized areas in the cerebral cortex or of their projection fibers.

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THE SPLEEN, HEMOGLOBIN AND ERYTHROCYTES IN NUTRITIONAL ANEMIA OF THE RAT

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The greatest interest in studies of nutritional anemia as induced in rats by the exclusive feeding of milk has centered about factors and substances effective in inducing recovery from the disorder, particularly the recovery of hemoglobin and erythrocytes. In the routine testing of foods for their hemoglobin regenerating values (1930–1931) based on the technique outlined by Waddell, Steenboch, Elvehjem and Hart (1), one of us (C. D. M.) observed that when certain foods were fed as supplements to milk the animals showed poor hemoglobin recovery and exhibited greatly enlarged spleens at autopsy. On the other hand animals used as controls and fed adequate amounts of liver or daily doses of 0.5 mgm. iron as ferric chloride and 0.01 mgm. copper as copper sulphate, showed complete recovery of hemoglobin and possessed normal sized spleens at autopsy.

A search of the literature on nutritional anemia of the rat at that time revealed no papers that reported an enlargement of the spleen to occur in that disorder. Since then a number of investigators have reported observations on the spleen in various types of anemia, only a few of which have a direct application to the present problem. Elvehjem and Sherman (2) have observed that when copper replaces iron as a supplement to milk, a decrease in the iron content and an increase in the size of the spleen result. Von Haam and Beard (3) have recently described the pathology of the spleen of rats with nutritional anemia. Macroscopically they found it to be small, pale, and soft in severe anemia. Microscopically there were fewer and smaller follicles and a decrease in the perifollicular zone of the follicles as well as a degeneration of the pulpar cells.

In 1933 we presented reports of our preliminary studies of the enlargement of the spleen (4) and its relation to various foods fed as a supplement to milk to induce recovery from anemia (5). The spleens of anemic animals and fully recovered animals were found to be of normal or nearly normal size. Enlarged spleens were found in animals that were recovering from the anemia. We concluded that the enlargement of the spleen was related to recovery and was of a temporary character. The present paper

deals with further experiments and observations on the enlargement of the spleen, some factors causing it and its relationship to recovery from nutritional anemia.

EXPERIMENTAL PROCEDURE. In some of the early experiments reported in this paper, young rats were placed on an exclusive diet of milk at the age of 24 to 28 days, but for most of the work anemia was produced by a slight modification of the method outlined by Elvehjem and Kemmerer (6). Milk used for the experiments, obtained from the University Farm, was collected in aluminum pails and poured directly into bottles. Analyses showed that the milk contained from 0.012 to 0.014 mgm. copper per 100 cc. and 0.12 mgm. iron per 100 cc. The animals were kept in galvanized iron cages and the milk was fed in glass dishes.

Several foods and inorganic substances were used as supplements to milk. The opihi tested for its hemoglobin regenerating properties was the limpet *Helcioniscus exaratus* or *H. argentatus*. Chemical analyses of the whole animal showed it to possess 0.0124 per cent iron and 0.000146 per cent copper. The calves' liver used in the experiments contained 0.0072 per cent iron and 0.00188 per cent copper. An analysis of the patent flour was not made. However, analysis of a patent flour was made at a later date, the results being similar to those given by Sherman (7), namely, 0.0013 per cent iron and 0.0002 per cent copper. Iron when fed alone as a supplement or in combination with copper was supplied by solutions of ferric chloride prepared from Baker's Analyzed $FeCl_3 \cdot 6H_2O$. The iron content of the solutions was checked colorimetrically. Copper was supplied by solutions of cupric sulphate prepared from Baker's Analyzed $CuSO_4 \cdot 5H_2O$. The copper content of the solutions was checked colorimetrically.

The blood samples were obtained from the rats' tails by the usual methods except that the tails were stabbed with fine pointed scissors instead of being clipped because they healed more readily after this technique. The hemoglobin determinations were made by the Newcomer method using a Klett colorimeter with a standardized Bausch and Lomb color plate. The erythrocyte and leucocyte counts were made by the usual technique using Thoma diluting pipettes and Levy-Hauser counting chambers. Differential leucocyte counts were made from smears stained with Wright's stain or Feemster's modification of Wright's stain.

At the end of the period of observation the animals were stunned by a blow on the head or lightly gassed, beheaded, and the blood drained from the body. The spleens were carefully removed, freed of all foreign tissue and weighed in tightly covered bottles. The spleens were fixed in formalin, Zenker's or Bouin's fixatives, sectioned at 5 to 7 micra and stained in Harris' hematoxylin and eosin or in Domonici's stain.

OBSERVATIONS AND RESULTS. This report of studies of nutritional anemia can be divided into three parts: 1, studies of the enlargement of

the spleen and hemoglobin regeneration, and their relation to diet; 2, studies of the relation of enlargement of the spleen to blood conditions when animals were fed copper and iron or combinations of the two in quantities known to have specific effects on recovery from anemia; 3, study of the histology of the spleens of animals fed the various diets.

Relation of hemoglobin regeneration and size of spleen to diet. The results of the feeding experiments are summarized in table 1. It presents averages of the body weight, hemoglobin levels, spleen weights and their per cent of the total body weight at the time of death of animals fed similar diets. Hemoglobin determinations were made at the end of each week of

TABLE 1

Relation of enlargement of the spleen to diet

DIET	AVERAGE OF SPLEEN AS PER CENT OF TOTAL BODY WEIGHT	AVERAGE WEIGHT OF SPLEEN IN GRAMS	AVERAGE GRAMS HEMOGLOBIN PER 100 CC. BLOOD	NUMBER OF ANIMALS
Milk only	0.31	0.21	3.3	17
Milk and 0.5 mgm. iron	0.31	0.23	3.9	9
Milk and 3 gms. flour*	0.61	0.74	3.4	6
Milk and 0.01 mgm. or 0.25 mgm. copper	0.43	0.52	5.5	9
Milk and 4 gms. opihi**	0.58	0.78	6.4	25
Milk, 4 gms. opihi and 0.01 mgm. copper	0.30	0.53	12.2	9
Milk and 4 gms. liver‡	0.26	0.49	12.8	13
Milk, 0.5 mgm. iron and 0.01 mgm. copper.	0.23	0.36	12.8	6
Total number of animals				94

* Three grams flour supplied, 0.042 mgm. iron, and 0.0006 mgm. copper. Based on average analyses.

** Four grams opihi supplied, 0.496 mgm. iron, and 0.00584 mgm. copper. Based on our analyses.

‡ Four grams liver supplied, 0.288 mgm. iron, and 0.075 mgm. copper. Based on our analyses.

supplementary feeding and the animals were continued on the supplementary foods until they had recovered or it was evident that further improvement would not occur. From the table it is evident that copper, iron and flour were ineffective or but slightly effective in producing hemoglobin recovery. Copper, supplementing iron and opihi, and liver produced a high grade of recovery. Foods which produced intermediate grades of recovery of hemoglobin were associated with the largest spleens, while those producing complete recovery or no recovery were associated with normal sized spleens. We have considered spleens of 0.3 to 0.35 per cent of the total body weight as normal.

A study of the size of the spleen of the animals referred to above, without reference to supplements fed, indicated that the enlargement of the spleen was associated with recovery from anemia. The greatest enlargement of the spleen was found to occur at hemoglobin levels of 6 to 8 grams per 100 cc. blood. Above these hemoglobin levels the spleen had regressed in size as indicated by a decrease of the actual weight of the organ and a decrease as its per cent of the total body weight. The enlargement of the spleen at hemoglobin levels of 6 to 8 grams and regression above those levels was also true of the individual groups of animals regardless of the supplement they had been fed, provided an incomplete type of recovery was produced.

Relation of the size of the spleen to blood conditions produced by iron and copper feeding. Since some relationship between the size of the spleen and recovery from anemia was clearly indicated by our early studies, a series of experiments was performed to prove this relationship. It was decided to use only copper and iron as supplements to milk as their effectiveness in curing anemia was known. The animals used in these experiments were divided into three groups, the first receiving iron as the only supplement, the second receiving copper as the only supplement, and the third receiving iron and copper administered simultaneously.

For the study of iron alone as a supplement to milk, a group of six animals was used, one of which was killed at the time the supplementary feeding was begun; while the remaining animals were fed 0.5 mgm. iron daily for a period of four weeks. Hemoglobin determinations, erythrocyte and leucocyte counts, and differential leucocyte counts were made at the time the supplementary feeding was begun and at the end of each week of feeding of the supplement. At the end of the four weeks all the animals were killed and the spleens removed, weighed and fixed for histological study. No changes of the blood elements studied occurred during the period of observation. Likewise there was no enlargement of the spleen, the average size of the spleen for the animals receiving iron being 0.1684 gram and 0.28 per cent of the total body weight.

The animals fed copper as the only supplement to milk were divided into two groups of five and seven animals. One animal of the first group and two of the second group were killed as controls at the time feeding of the supplement began. The remaining four animals of the first group were given daily doses 0.01 mgm. copper, while the remaining five animals of the second group were given daily doses of 0.25 mgm. copper. Both groups were given the copper for a period of four weeks and blood studies were made as in the iron series. The results for the two groups were similar except that the response was larger and more rapid when 0.25 mgm. copper was given then when 0.01 mgm. was fed. The results for the second group are summarized in table 2. It shows that erythrocyte recovery was

more rapid and more complete than hemoglobin recovery and that animals which had most nearly approached the normal blood conditions, particularly as regards the erythrocytes, possessed the smallest spleens. The results of this experiment may be taken to indicate that enlargement of the spleen was more definitely related to recovery of erythrocytes than to recovery of hemoglobin.

The animals fed copper and iron were divided into two groups of eleven animals each. Three of the first group and two of the second group were killed as controls at the time the feeding of the supplement was begun. The remaining animals were all given 0.5 mgm. iron daily; the first group

TABLE 2

*Results of feeding 0.25 mgm. copper daily for four weeks**

ANIMAL NUMBER	HEMOGLOBIN IN GRAMS PER 100 CC. BLOOD	NUMBER OF ERYTHROCYTES PER CU. MM. BLOOD	NUMBER OF LEUCOCYTES PER CU. MM. BLOOD	WEIGHT OF SPLEEN IN GRAMS	SPLEEN AS PER CENT OF TOTAL BODY WEIGHT
2017 Control	3.7	3,850,000	3,800	0.23	0.33
2016 Control	5.5	5,020,000	4,800	0.23	0.33
2013	4.5	4,210,000	3,300		
	5.3	6,700,000	8,400	0.61	0.42
2011	4.1	4,207,000	3,350		
	5.5	7,200,000	8,360	0.72	0.45
2012	3.3	3,840,000	3,020		
	7.0	7,690,000	6,800	0.66	0.53
2015	3.9	4,575,000	2,928		
	7.9	8,740,000	8,500	0.37	0.27
2014	3.4	3,270,000	3,350		
	6.7	9,190,000	8,100	0.39	0.26

* First figure following number of animal for beginning; second for end of supplement feeding.

in addition receiving 0.01 mgm. copper daily, while the second group received 0.25 mgm. copper daily. Instead of feeding the supplement for a definite period of time, one animal was killed for each gram increase of hemoglobin above the anemic level. Blood studies were made at the time that the feeding of the supplement was begun, at the end of each week of feeding and at the time that the animals were killed. The response of the animals of the two groups to the supplementary feeding was similar in every respect except in rate of recovery, the animals of the second recovering more rapidly than the animals of the first group. The results for the second group are summarized in table 3. The largest spleens, 0.9 to 1.1 per cent of the total body weight, were associated with hemoglobin levels of

5 to 8 grams and erythrocyte counts of four million. A regression of the size of the spleen, as shown by a decrease in the actual weight and per cent of the total body weight, occurred above these levels and was associated with a rapid increase of erythrocytes. The differential leucocyte counts showed very little change in the proportion of the different types of leucocytes, though the total number had increased greatly. In a few instances normoblasts were found to constitute 20 to 25 per cent of the total

TABLE 3

*Results of feeding 0.25 mgm. copper and 0.5 mgm. iron daily**

ANIMAL NUMBER	HEMOGLOBIN IN GRAMS PER 100 CC. BLOOD	NUMBER OF ERYTHROCYTES PER CU. MM. BLOOD	NUMBER OF LEUCOCYTES PER CU. MM. BLOOD	WEIGHT OF SPLEEN IN GRAMS	SPLEEN AS PER CENT OF TOTAL BODY WEIGHT
2152 Control	2.4	2,205,000	1,775	0.20	0.31
2148 Control	3.5	2,925,000	3,125	0.20	0.35
2142 (2)	3.5	3,515,000	10,575		
	4.7	3,595,000	23,150	0.53	0.69
2141 (2)	3.7	3,505,000	17,000		
	5.5	4,130,000	24,500	0.68	0.97
2153 (2)	3.9	2,790,000	3,300		
	6.7	4,265,000	13,450	0.76	1.10
2154 (7)	3.9	1,745,000	6,650		
	7.9	4,845,000	7,400	0.87	1.08
2143 (4)	3.6	4,075,000	10,575		
	8.5	6,135,000	6,525	0.50	0.69
2151 (8)	2.4	2,265,000	4,825		
	9.9	6,580,000	9,100	0.62	0.68
2144 (6)	3.2	2,875,000	10,725		
	10.5	4,880,000	5,450	0.37	0.53
2150 (18)	4.5	4,080,000	8,700		
	11.5	7,390,000	9,000	0.58	0.41
2155 (18)	4.2	2,420,000	3,675		
	12.6	9,180,000	7,676	0.38	0.36

* Figures in parentheses indicate number of days the animal received supplement. First figure following number of animal for beginning of supplement feeding; second figure for end of feeding.

nucleated cells though in most cases they were found to constitute less than 5 per cent of the nucleated cells.

Histology of the spleen. The spleens of animals in different stages of recovery from anemia differ greatly in histological structure and it can be shown that the histological changes of the spleen, regardless of the supplementary diet fed, are associated with recovery. Histological structures characteristically appearing after the feeding of a certain diet are structures

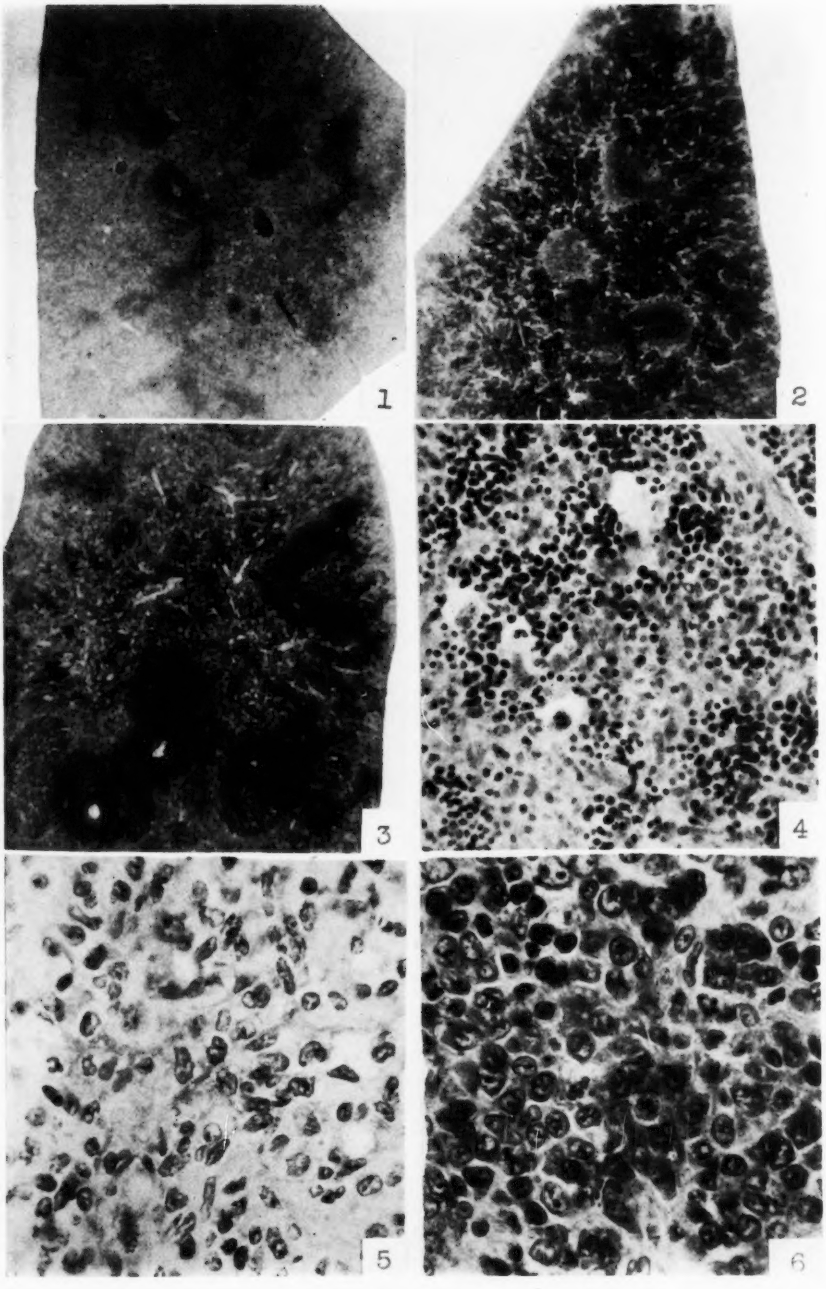

PLATE I

actually associated with the stage of recovery produced by that diet and a similar histological structure can be found in the spleen of animals in the same stage of recovery though fed a different diet.

The spleens of anemic or control animals had undergone degeneration and disorganization. In severe anemia they showed a decrease in the white pulp, the outer zone and a large portion of the inner zones of the Malpighian corpuscles and the outer portions of the periarterial pulp as well having disappeared. As a result the red pulp appeared to have increased, the proportion of white to red pulp being usually 1 to 8 or 10. Only pulpar cells and erythrocytes were present in the red pulp areas imparting a clear and open appearance to these areas. The pulpar cells themselves showed some signs of degeneration, and mitotic figures could not be found in sections of these spleens indicating a cessation of growth and of production of blood corpuscles. The changes of the spleen were less marked in moderate anemia, the outer zone of the Malpighian corpuscle having disappeared while the inner zone was still intact and well defined. The red pulp areas were similar to those of the spleen of severely anemic animals except that occasional basophilic stem cells and fairly numerous normoblasts were present in these areas. There was no indication of cell multiplication.

The simultaneous feeding of copper and iron as supplements to milk caused a regeneration of the spleen. Well defined periarterial pulp areas and complete and well organized Malpighian corpuscles were found at hemoglobin levels of 6 to 8 grams' hemoglobin and erythrocytes number-

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Plate I. Explanation of figures.

1. Spleen of animal fed iron for 4 weeks (2145). Note the small size of the Malpighian corpuscles and periarterial pulp areas and the uniformly clear appearance of the red pulp areas. Hemoglobin, 3.1 grams; erythrocytes, 2,870,000; leucocytes, 2,700. × 30. This appearance is also characteristic of the spleen of control animals that received milk only.

2. Spleen of animal fed iron and copper for 7 days (2005). Note the small size of Malpighian corpuscles and periarterial pulp, though otherwise well organized, and the large number of erythropoietic centers in the red pulp areas. Hemoglobin, 6.0; erythrocytes, 4,960,000; leucocytes, 2,750. × 30.

3. Spleen of animal fed copper and iron for 38 days (2021). Note the large size and the well developed character of the Malpighian corpuscles and periarterial pulp and the small number of erythropoietic centers in the red pulp areas. The red pulp areas are composed largely of normoblasts. Hemoglobin, 11.5; erythrocytes, 8,160,000; leucocytes, 7,100. × 30.

4. Portion of spleen of animal fed iron and copper (2150). Note the small number of basophilic stem cells and large number of maturation stages of erythrocytes. Hemoglobin, 11.5; erythrocytes, 7,390,000; leucocytes, 9,000. × 310.

5. Portion of spleen of anemic control animal (2004). Note the open and clear appearance of the red pulp areas. Hemoglobin, 2.5; erythrocytes, 3,900,000; leucocytes, 5,000. × 660.

6. Portion of spleen shown in figure 2. Note the basophilic stem cells. × 660.

ing four million. Actively mitotic germinal centers had appeared in the corpuscles indicating a resumption of cell multiplication. The greatest histological change, at the erythrocyte and hemoglobin levels referred to above, appeared in the red pulp areas, the entire area having been transformed into erythropoietic centers. As a result the spleens at the time of greatest enlargement possessed red pulp areas composed largely of basophilic stem cells. At slightly higher hemoglobin and erythrocyte levels, erythropoietic differentiation and megakaryocytes appeared in the red pulp areas, while the stem cells tended to decrease in number. Thus at hemoglobin levels of 9 grams and erythrocytes numbering 6 to 7 million, the spleen usually possessed only a few stem cells scattered in nests throughout the red pulp areas while numerous normoblasts and fairly numerous magakaryocytes were present. The stem cells, normoblasts and megakaryocytes disappeared on further improvement, the megakaryocytes being the last to disappear.

The spleens of animals fed iron alone as a supplement to milk possessed the same histological structure as the spleens of severely anemic animals. Iron, therefore, does not stimulate splenic regeneration or cell production. Copper when fed alone under the conditions of our experiments caused a regeneration of the white pulp of the spleen, the parts of the white pulp being reconstructed in the same manner as that produced by the simultaneous feeding of copper and iron. It also caused the appearance of basophilic stem cells and active erythropoiesis in the red pulp areas. The basophilic stem cells were most numerous in the early stages of recovery, normoblasts and megakaryocytes in the later stages. Stem cells, normoblasts and megakaryocytes tended to disappear on further improvement as shown by the spleen of animal 2014 (table 2) which had recovered to the extent that it possessed nine million erythrocytes and 7.0 grams hemoglobin. In this case, where hemoglobin recovery and erythrocyte recovery had proceeded at an unequal rate, the relation of the enlargement and regression in size of the spleen, with their attendant histological changes to erythrocyte recovery, is quite clearly shown.

The animals that were fed liver as a supplement to milk were killed when they had fully recovered or nearly recovered from nutritional anemia. Histologically the spleens of liver fed animals with hemoglobin of 10 to 12 grams possessed well defined white pulp and red pulp lacking in stem cells, normoblasts or megakaryocytes. However, the spleens of animals with hemoglobin of 9 grams possessed occasional stem cells and a few normatoblasts, indicating that in the early stages of recovery active erythropoiesis had occurred. Opihi also stimulated white pulp regeneration and hematopoiesis and histologically the spleens of animals fed this diet conformed to the structure of spleens of animals of similar stage of recovery but fed iron and copper. The spleens of animals fed flour were as a rule very large,

possessed fairly well defined and organized white pulp but otherwise were characterized by the presence of extremely large numbers of basophilic stem cells in the red pulp areas.

DISCUSSION. The most important observation recorded in this paper is that of the character and relationships of the enlargement of the spleen during recovery from nutritional anemia. An enlargement of this organ has previously been shown to occur in isolated cases in nutritional anemia by Elvehjem and Sherman (2), in two instances as the result of copper feeding by Perla and Marmorston-Gottesman (8), and to occur regularly in recovery from nutritional anemia by Hamre and Miller (4, 5). It is significant that the enlargement and erythropoietic changes of the spleen occur in the early stages of recovery and that it regresses in size and the erythropoietic processes disappear in the later stages of recovery. The enlargement is of the nature of a compensatory hypertrophy caused by the formation of a large number of basophilic stem cells, while the decrease in the size of the organ is associated with the differentiation of these cells into erythrocytes and the discharge of the latter into the blood stream. Also, if recovery is complete, splenic enlargement is temporary; while if it is incomplete it apparently continues without regression, possibly due to the failure or retardation of differentiation of erythrocytes. It follows, therefore, that under the conditions of our experiments, the spleen is an important factor in recovery from anemia. From unpublished data we have learned that the bone marrow does not respond as readily to erythropoietic stimulation as does the spleen under those experimental conditions.

The enlargement and erythropoietic response of the spleen to various supplementary substances has an important bearing on the question of the effectiveness of these substances as curative agents in nutritional anemia. The observation that the spleen passes through a complete cycle of histological change associated with complete hemoglobin and erythrocyte recovery when copper and iron are fed simultaneously gives additional confirmation to previous observations of the effectiveness of these substances as curative agents. It thus confirms the observations of Hart and Steenboch and their co-workers (1, 9, 10, 11, 12, 13, 14), of Beard and Myers and their co-workers (15, 16, 17, 18, 19, 20), of Mitchell and Miller (21) and others. Also the lack of response of the spleen as well as the failure of recovery of hemoglobin and erythrocytes when iron was fed alone as supplement to milk, lends support to the views of the Wisconsin workers of the ineffectiveness of this substance as a curative agent when fed alone. The low grade of hemoglobin recovery resulting from the feeding of copper alone as a supplement to milk conforms to the generally accepted view that iron must be present for the formation of hemoglobin. The marked increase of erythrocytes and the erythropoietic response of the spleen to copper feeding lends support to the views of Stein and Lewis (22, 23) and

Schultze, referred to by Stein and Lewis, that copper exerts an influence upon recovery independent of iron, namely, that of stimulation of cell production.

Of the supplementary foods studied, those possessing adequate quantities of copper and iron elicited the same response of the spleen as adequate quantities of inorganic salts supplying these elements. Thus liver supplying adequate copper and iron was found to produce a complete and typical response of the spleen and complete recovery of hemoglobin and erythrocytes. The same was true for opihi except that hemoglobin recovery was less complete, copper in this case apparently being inadequate for hemoglobin recovery was complete when this element was added. Flour possessing small quantities of both iron and copper caused the spleen to enlarge and stimulated cell production, which was not sustained, as well as only slight formation of hemoglobin.

We suggest on the basis of our observations that the hematopoietic processes and functions of the spleen of young animals are suppressed by an exclusive diet of milk, and that these processes and functions are resumed when adequate diets are supplied. On the basis of the experiments here reported an "adequate diet" for the normal functioning of the spleen is one that supplies sufficient copper and possibly iron, either in inorganic form or in food combinations. Additional, carefully controlled experiments may demonstrate that of the two elements, copper alone is concerned in the cytogenic function of the spleen.

CONCLUSIONS

1. The spleen is disorganized, to some extent degenerated, and is not active in the production of blood elements in severe nutritional anemia of the rat.

2. The spleen enlarges temporarily during the period of recovery from nutritional anemia when adequate quantities of copper and iron are fed either in the form of inorganic salts or in natural foods. During the period of enlargement, the spleen is actively erythropoietic and therefore concerned in the increase in number of erythrocytes.

3. When fed alone as a supplement to milk, iron does not cause an enlargement of the spleen, an increase of erythrocytes, nor a marked increase of hemoglobin.

4. When fed alone as a supplement to milk, copper causes an enlargement of the spleen, and an active erythropoiesis associated with a recovery of erythrocytes but does not cause a complete recovery of hemoglobin.

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RHYTHMIC CHANGES IN THE FETAL LIVER AFTER FEEDING

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Experimental evidence has conclusively demonstrated a cyclic activity, following feeding, in some of the better known constituents of the animal liver. These cyclic changes have been attributed by some workers (1, 2) to an intrinsic periodicity within the organ itself although the results of a recent study in this laboratory (3) seemed to indicate that these changes were the result of factors associated wholly with feeding.

Goodwin and Higgins (4) recently have studied the livers of pregnant rats with respect to weight and glycogen and water content following a single feeding. They learned that the curves describing the changes in these constituents of the liver for twenty-four hours following feeding were essentially like those that described the changes for nonpregnant animals. They found, however, that whereas the bimodal curves of these changes for both pregnant and nonpregnant animals were in general alike, the initial mode always appeared more rapidly, following the taking of food in the case of the pregnant animal than it did with the nonpregnant animal.

Since rhythmic changes occurred in maternal livers during pregnancy, we were interested to know whether similar changes took place in the fetal livers, and whether the curve of these changes was likewise bimodal.

Aron (1) studied the glycogenic functions of the fetal livers of sheep, guinea pig, and man. He found that the glycogenic function was assumed by the liver at the time the interstitial portion of the pancreas developed and he expressed the belief that the transference of the glycogenic function from the placenta to the liver was controlled by some endocrine activity.

Methods of experimentation. Rats from our colony which were of an original Wistar strain were used for this study. They were allowed free access to our adequate rat diet and to water during breeding and early pregnancy; however, one week before term they were isolated and were given food and water only during the hours from 9 to 11 each morning. In this way they were trained to take the necessary food and water for twenty-four hours at this single two-hour period.

Early in our study we attempted to predict the time of parturition from the appearance of spermatozoa in the vaginal smears that were made each

morning. As a result of apparent variations in the time relations of ovulation and insemination, however, we encountered considerable variations in the onset of term, and we finally resorted to the method of palpation as one sufficiently reliable to enable us to predict the approximate time of delivery. We wished to examine these fetal livers at as near term as possible; whether this was the twentieth or twenty-first day of pregnancy was not of particular significance.

Sixty-six pregnant, full-term rats were used in this study. It was obviously impossible to secure so large a number of animals which would deliver at the same time. Accordingly, it was necessary to utilize all animals as they came to term, to feed them at corresponding times of the day, that is, from 9 to 11 a.m. and to kill them at such times after feeding as we wished to examine both the maternal and fetal livers. Eleven of the sixty-six animals were killed before the morning feeding period after a fast of twenty-four hours; these furnished the control data on hepatic weight and water and glycogen content with which those data derived from all other animals were compared. A regression formula was developed from these control data whereby we were able to predict or to estimate the weights of the livers of all animals from the weights of the bodies at 9 a.m. just before feeding. The formula employed was L.W. = 0.017 B.W. + 2.318 gram, L.W. being weight of liver and B.W. the weight of the body.

Following the morning feeding, five animals were killed every two hours from 11 a.m. on one day to 7 the following morning. Thus eleven groups of five animals each were killed. The changes in the weight of the animal and in the weight of the maternal liver and its hepatic glycogen and water for each two-hour period were computed from the weights at 9 a.m. before feeding. The average change in the amounts of these constituents for the five animals killed at each succeeding interval was the average change recorded for the time that had elapsed since feeding. The statistical method, we admit, has its limitations and its errors, but it was obviously impossible to examine a single animal at these successive intervals after feeding for such data as we wished to assemble.

The changes in weight of the fetal liver and in its water and glycogen content were not so easily computed. Litters were not of comparable number or size, and it was obviously impossible to determine the weight of a fetus at 9 a.m. before the mother was fed. Thus changes in the weight of fetal livers, in grams increase or decrease, could not be determined, and we were restricted to a consideration of percentage changes in the fetal hepatic glycogen. Determinations of the water content of fetal livers, in per cent, were made; however, such figures are not sufficiently adequate to portray the changes going on and they have not been included in this report.

All animals were killed by severing the jugular vessels. A median, ventral incision was quickly made and a piece of maternal liver, weighing about 500 mgm., was snipped off from the presenting left lobe and was placed immediately in a freezing solution of alcohol and carbon dioxide ice. The specimen was then weighed, placed in a 60 per cent solution of potassium hydroxide, and boiled for one and a half hours. The estimation of glycogen was performed according to the Pflüger method.

Four fetuses were removed from the uterus immediately after excising the piece of maternal liver. A cross incision was made in the abdomen of each fetus and, by means of dorsiflexion of the body, the entire fetal liver was thereby presented. The entire fetus, with its protruding liver, was then immersed in the freezing solution for about a minute and a half,

TABLE 1

Changes in weight of maternal liver and in amount of maternal hepatic glycogen and water

TIME		CHANGE IN WEIGHT FROM THAT AT 9 A. M., GM.		
Actual	Elapsed since feeding	Liver	Glycogen	Water
	hours			
11:00 a.m.	2	1.551 ±0.154	0.1154 ±0.0067	1.529 ±0.037
1:00 p.m.	4	2.645 ±0.421	0.2097 ±0.023	2.149 ±0.345
3:00 p.m.	6	2.037 ±0.165	0.3805 ±0.016	1.572 ±0.032
5:00 p.m.	8	2.128 ±0.243	0.5654 ±0.046	1.583 ±0.076
7:00 p.m.	10	1.448 ±0.171	0.356 ±0.037	0.903 ±0.202
9:00 p.m.	12	2.278 ±0.350	2.2344 ±0.016	1.345 ±0.348
11:00 p.m.	14	2.306 ±0.347	0.266 ±0.002	1.748 ±0.152
1:00 a.m.	16	2.448 ±0.474	0.290 ±0.037	1.770 ±0.221
3:00 a.m.	18	2.759 ±0.269	0.280 ±0.012	2.274 ±0.322
5:00 a.m.	20	2.466 ±0.372	0.238 ±0.040	1.555 ±0.299
7:00 a.m.	22	1.717 ±0.173	0.047 ±0.002	1.207 ±0.084

or until frozen, whereupon the liver could readily be removed in toto. After weighing each liver, determinations of glycogen were made according to Pflüger's method. An additional portion of the maternal liver, and four additional fetal livers, were then weighed and used for the percentage determinations of water content. These specimens were dried in an incubator until the weights became constant. The total weights of maternal livers, including the two portions that were used for determinations of glycogen and water, were also computed.

Experimental observations. *Maternal liver.* The changes in weight of the maternal liver and in the amounts of maternal hepatic glycogen and water during the twenty-two hours following a single feeding are condensed in table 1. The results are not significantly different from those

of Goodwin and Higgins, and the curve of these changes is again found to be bimodal. There are differences in the time of appearance of the modes, but in general the curves of these changes in the constituents of the liver, as revealed in these two studies on pregnant animals, are comparable.

Fetal liver. As was indicated before, actual changes in the weights of water or glycogen in the fetal liver during the post-feeding period of the mother could not be determined without a regression formula to compute the changes in total hepatic weight. Changes in glycogen and water in the livers of adult animals have hitherto been determined on the basis of the total hepatic weight. Since it was obviously impossible to record increases in weights of fetal livers during the cycle following feeding, we were restricted to a consideration of percentage computations only.

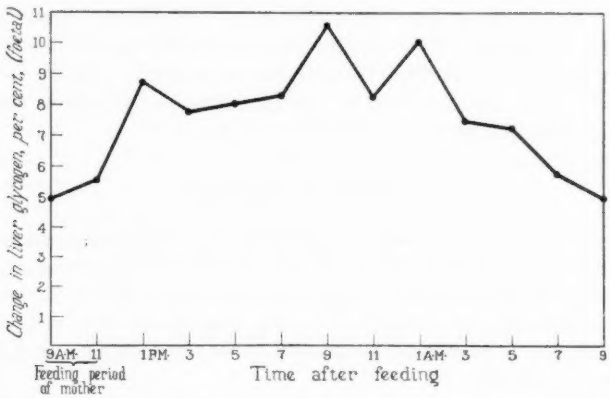

Fig. 1. Rhythmic changes in fetal liver after feeding

As far as the percentage of water in these fetal livers is concerned, little need be said. This component of water varied from 80 to 85 per cent of the total weight of the liver, irrespective of the time the fetuses were examined, and thus it did not seem to bear any significant relation to the time of feeding.

Glycogen values in per cent, however, were more informative, and the curve of the changes in these values is given in figure 1. The percentage of glycogen in the fetal liver, even after a twenty-four hour fast of the mother, was always relatively high. On the basis of determinations of these values for the livers of forty-four fetal rats, made at 9 a.m. after withholding food from the mothers for twenty-four hours, glycogen constituted 4.95 per cent of the total weight of the fetal liver.

Glycogen determinations were made on twenty fetal livers, taken from the fetuses of five adult rats, four fetuses from each adult every two hours

until 7 a.m. the following morning. The curve describing these changes in percentages of glycogen in fetal livers (fig. 1) is not as definitely bimodal as that depicting changes in total weight of maternal livers; yet there are some indications in that direction. At 11 a.m., at the end of the feeding period, an increase of 0.4 per cent in glycogen was encountered, whereas at 1 p.m., at the time of the initial mode on the curve describing changes in weights of maternal livers we found a percentage of glycogen in fetal livers of 8.75. This is an increase, during the period, of 3.8 per cent in the amount of glycogen present. A slight drop in the percentage of fetal hepatic glycogen from the reading at 1 p.m. occurred at 3, 5, and 7 p.m. This was followed at 9 p.m., when the total weight of the maternal liver was again high, by a second increase in the amount of glycogen in the fetal liver. The average percentage of glycogen in the twenty fetal livers at 9 p.m. was slightly more than double the percentage found at 9 a.m. twelve hours before. There was a transient fall in the percentage of glycogen at 11 p.m., but the rise at 1 a.m. was nearly as high as that recorded at 9 p.m. From then on the percentage of glycogen recedes to the level established at 9 a.m. before feeding.

COMMENT AND SUMMARY

As far as the maternal liver is concerned, this study demonstrates again the existence of a cyclic activity in the deposition of some of the better known hepatic constituents. It corroborates the work of Goodwin and Higgins in that there appears to be a more rapid metabolism of these constituents in the liver during pregnancy than was found to occur among nonpregnant animals.

Although we have been unable to measure the actual increase in the amounts of these constituents formed in the fetal liver, yet by means of percentage determinations we have been able to show that the proportion of glycogen in fetal livers increased and then decreased following feeding of the mother.

The curve representing the changes in the percentage of glycogen, points of which were plotted at intervals of two hours throughout the period of twenty-two hours following feeding, shows a gradual increase from the proportions present at 9 a.m. to an amount approximately twice as high at 9 p.m. Thereafter, with some fluctuations, the trend of the curve is downward, and at 9 the following morning a mean percentage of glycogen of 5.0 was again encountered.

Aron has demonstrated that the development of physiologic potency in the fetal liver, as far as glycogen is concerned, is correlated with the appearance of islets in the pancreas. Early in development, the placenta serves to regulate fetal metabolism of glycogen; however, with the growth

of the islets of Langerhans, this function is gradually transferred to the liver and the placental glycogen steadily decreases in amount until term.

Fetal hepatic glycogen in proportion to the weight of the liver was higher than maternal liver glycogen, both before and after feeding. During a twenty-four hour fast, maternal hepatic glycogen was reduced to an amount constituting but 0.29 per cent of the total hepatic weight, whereas much higher values were maintained in the fetus. After food had been withheld for twenty-four hours, when the percentage of glycogen in the maternal liver had dropped to 0.29, a mean percentage of glycogen in the fetal liver of 4.95 was recorded. Immediately on feeding, when glucose appeared in the maternal blood stream, percentages of glycogen at once began to rise in the fetal liver. Glucose probably passed the placental barrier and served to increase the already large store of glycogen present in the fetal liver. Whereas hepatic glycogen increased in the maternal liver during the period after feeding, from 0.29 per cent of the hepatic weight to 3.1 per cent, the percentage increase in the fetal liver ranged from 4.95 to 10.60.

The curve of these percentage changes is not particularly bimodal in character, but neither is that depicting the total changes of glycogen in the maternal liver. The peak in the amount of glycogen stored in the maternal liver was reached at 5 p.m., whereas the high point on the curve for percentage of fetal hepatic glycogen did not occur until 9 p.m. Percentage changes are not comparable to actual changes in weight, and yet because of the similarity in weights of the fetuses and of the fetal livers we are inclined to believe that were we able to record total changes in fetal hepatic glycogen during the cycle, a somewhat comparable curve would result.

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THE INFLUENCE OF GLYCINE ON THE EXCRETION OF CREATINE AND CREATININE

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In 1905 Folin (1) demonstrated that the creatinine elimination for any one individual on a meat free diet is an approximately constant quantity independent of marked changes in the nitrogen output. Since that time the literature has been filled with descriptions of attempts to demonstrate precursors of both creatine and creatinine. The results have been suggestive but quite contradictory, and no specific precursor has been definitely established. More recently the reports by Brand, Harris, Sandberg and Ringer (2) of an increased creatine excretion after feeding glycine to patients who had progressive muscular dystrophy, and by Thomas, Milhorat and Technor (3) of the therapeutic use of glycine have stimulated renewed interest in the metabolism of creatine and creatinine. Of the amino-acids studied by Brand, Harris, Sandberg and Ringer (2), glycine was the only one to cause any marked increase in creatine excretion. Many investigators have since confirmed the immediate increase in creatine after the administration of glycine to patients who had progressive muscular dystrophy, although there seems to be considerable variation in the results reported for the changes occurring over longer intervals. Brand and Harris (4) and Reinhold, Clark, Kingsley and Custer (5) have found that the higher level of creatine excretion produced by glycine was maintained as long as the subjects continued to take glycine. Thomas, Milhorat and Technor (3), on the other hand, have observed a subsequent drop in the creatine excretion and their results have been confirmed by Tripoli and Beard (6), Kostakow and Slauck (7), Mettel and Slocum (8), and Sullivan and Hess (9).

According to Milhorat (10), glycine produced an increase in the excretion of creatine only in disturbances which are primarily muscular. In general, however, the results reported in the literature concerning patients other than those who had progressive muscular dystrophy are so conflicting that it is difficult to draw any general conclusions concerning the influence of glycine on the creatine and creatinine metabolism. Likewise, the small amount of data so far reported concerning strictly normal subjects is of a similar contradictory nature.

In the present paper are the results of an extended investigation of the influence of glycine on the excretion of creatine and creatinine by more than 100 subjects, not only subjects who had progressive muscular dystrophy and myasthenia gravis, but also a miscellaneous group including a large number of individuals concerning whom there were observed no striking abnormalities in the creatine and creatinine metabolism.

Experimental conditions. The excretion of creatine and creatinine for each twenty-four hours was determined for at least two days previous to feeding of glycine, or for a longer control period if possible. Glycine was then administered in amounts varying from 5 to 40 grams daily, divided into three doses, one taken after each meal. After administration of glycine had been started, the daily determinations were continued as long as the subject was under observation, generally for at least two weeks, and in many cases for periods of from one to six months. The daily excretion of creatine and creatinine for periods of, usually, seven days were averaged and the values thus obtained form the basis of the data in this paper. No attempt was made to restrict the subjects to creatine-free diets because such diets did not seem desirable for some of the severe muscular disturbances under treatment. The constant average daily creatine excretion observed after equilibrium had been established on any given regimen suggests that the creatine intake was on the average approximately constant. Preformed creatinine was determined by Folin's colorimetric method, using creatinine zinc chloride as the standard. For the total creatinine determinations, creatine was converted to creatinine either by Folin's (11) method of boiling with picric acid or by Benedict's (12) method of heating with hydrochloric acid. The majority of the determinations were made by the first of these two methods. The addition to the urine of 10 to 20 mgm. of glycine or sodium glutamate for each milligram of creatinine was found to be without influence on the creatinine and creatine determinations. In some cases because of the possibility that heating with acid may have generated chromogenic substances from sources other than creatine, further indirect evidence that the changes observed as the result of feeding glycine were attributable to creatine was obtained by adsorption experiments with Lloyd's reagent. The method used was the modification of Gaebler's (13) procedure described by Shannon, Jolliffe and Smith (14). The values for creatinine and creatine, determined by the direct or by the adsorption method were in reasonable agreement.

The influence of glycine on the excretion of creatine. The subjects studied are considered in three groups. The first is a miscellaneous group of fifty subjects, as follows: two were normal individuals; twenty-eight had chronic nervous exhaustion; nine, arthritis; two, myotonic dystrophy; two, colitis, and one each, progressive muscular atrophy,

dermatomyositis, myotonia congenita, dystonia musculorum deformans, chronic poliomyelitis with bulbar palsy, encephalitis, and menorrhagia with obesity. The second is a group of thirty subjects who had myasthenia gravis, and the third, twenty-three subjects afflicted with progressive muscular dystrophy. The extent of the creatinuria in these three groups, before administration of glycine, and the maximal daily creatine excretion attained during the administration of glycine, is summarized in table 1. Somewhat more detailed results of the feeding of glycine to a group of subjects who normally excreted 0.02 gram creatine nitrogen or less are shown in table 2. Similar data have been obtained for all the individuals under observation but the subjects represented in table 2 have been selected because of their normally low creatinuria.

From tables 1 and 2 it is evident that before the administration of glycine many of the subjects in both the miscellaneous and the myasthenia gravis groups excreted only small amounts of creatine. Thirty of the thirty-six males excreted less than 0.05 gram creatine nitrogen. Among the females the incidence of marked creatinuria was higher, twenty of forty-six excreting 0.05 gram creatine nitrogen or more. The tendency of normal women to excrete creatine has also been frequently noted. The creatinine excretion, as indicated by the creatinine coefficient, is seen to be within normal limits for all the subjects listed in table 2 and this was true for the majority of subjects in groups one and two. It appears, therefore, that the creatine-creatinine metabolism of these subjects did not deviate markedly from that of a similar group of normal individuals on an unrestricted diet. On the other hand, the creatine-creatinine metabolism of the subjects with progressive muscular dystrophy was far from normal. As table 1 shows, before the administration of glycine 78 per cent of these subjects were excreting more than 0.10 gram creatine nitrogen. The creatine excretion of these patients was usually more than 50 per cent of their total creatine and creatinine nitrogen. Furthermore, their creatinine excretion was definitely less than that of normal individuals of comparable age.

The administration of glycine was followed by a definite increase in the creatine excretion of the majority of the subjects, as is shown in table 1. Eighty per cent of the subjects in groups 1 and 2 and all of those in group 3 excreted more creatine after administration of glycine than before. This is further illustrated for many of the subjects of groups 1 and 2 by the more detailed data selected for inclusion in table 2. Twenty-three of the twenty-eight subjects represented in this table excreted 0.05 gram or more of creatine nitrogen after administration of glycine, a definite increase over their control level of 0.02 gram or less. As has been stated, the figures given represent average values, so that the increases observed cannot be considered as merely temporary and attributable possibly to an abnormally high creatine intake on some one day.

TABLE 1

Distribution of subjects as regards creatine excretion before, and maximal creatine excretion after, beginning administration of glycine

BEFORE GLYCINE		AFTER GLYCINE				
		Creatine N 0.00–0.05	Creatine N 0.05–0.10	Creatine N 0.10–0.20	Creatine N 0.20–0.30	Creatine N 0.30–0.50
Miscellaneous. Group 1						
Creatine N*	0.00–0.05					
Males	14	4	7	3		
Females	19	6	7	6		
Creatine N	0.05–0.10					
Males	2		2			
Females	11		1	5	4	1
Creatine N	0.10–0.20					
Males	1					1
Females	3			3		
Myasthenia gravis. Group 2						
Creatine N	0.00–0.05					
Males	16	1	5	7	3	
Females	7		4	2	1	
Creatine N	0.05–0.10					
Males	2		1	1		
Females	1			1		
Creatine N	0.10–0.20					
Males	1				1	
Females	4			2	2	
Muscular dystrophy. Group 3						
Creatine N	0.00–0.05					
Males						
Females	1				1	
Creatine N	0.05–0.10					
Males	2			2		
Females	2			2		
Creatine N	0.10–0.20					
Males	12				7	5
Females	2				1	1
Creatine N	0.20–0.30					
Males	3				1	2
Females						
Creatine N	0.30–0.40					
Males	1					1
Females						

* Values for creatine N, throughout the table, are in grams per 24 hours.

TABLE 2

The influence of glycine on the daily excretion of creatine and creatinine

SUBJECT	DIAGNOSIS	AGE, YEARS	WEIGHT, KGM.	GLYCINE DAILY, GRAMS	DAYS	PREFORMED CREATININE NITROGEN, GRAMS		CREATINE NITROGEN, GRAMS		PREFORMED CREATININE COEFFICIENT, MGM. NITROGEN PER KGM.
						Before	After	Before	After	Before
	Males									
1	Myasthenia gravis	42	74.0	30	32	0.49	0.47–0.55	0.02	0.11	6.6
2	Myasthenia gravis	56	78.0	30	17	0.57	0.56–0.59	0.00	0.02–0.03	7.4
3	Chronic nervous exhaustion	49	63.2	25–30	28	0.48	0.48–0.53	0.02	0.01–0.03	7.6
4	Myasthenia gravis	63	79.0	30–40	35	0.62	0.58–0.72	0.02	0.02–0.06	7.8
5	Chronic poliomyelitis	36	54.1	5–10	37	0.43	0.43–0.43	0.02	0.01–0.02	7.9
6	Myasthenia gravis	28	66	20–40	33	0.53	0.56–0.60	0.02	0.02–0.10	8.0
7	Biologic inferiority	31	79.9	20	8	0.66	0.68	0.02	0.09	8.3
8	Effort syndrome	34	85.3	30	24	0.71	0.70–0.75	0.02	0.02–0.08	8.3
9	Myasthenia gravis	33	69	30–40	40	0.58	0.59–0.72	0.01	0.03–0.11	8.4
10	Myasthenia gravis	48	78	30–35	77	0.67	0.65–0.77	0.02	0.01–0.12	8.6
11	Myasthenia gravis	40	81.1	20–30	41	0.73	0.69–0.79	0.02	0.04–0.15	9.0
12	Exhaustion	19	65.1	30–40	15	0.60	0.62–0.63	0.00	0.02–0.02	9.2
13	Myasthenia gravis	19	48	30–35	33	0.47	0.47–0.47	0.02	0.11–0.15	9.7
14	Chronic nervous exhaustion	38	56.9	30	29	0.61	0.61–0.63	0.02	0.03–0.05	10.3
15	Fatigability	30	81.8	30	9	0.89	0.78–0.79	0.01	0.04–0.05	10.9
	Females									
16	Myasthenia gravis	40	67	20–30	26	0.36	0.35–0.37	0.02	0.05–0.14	5.4
17	Mild exophthalmic goiter	20	46.2	30	26	0.26	0.27–0.31	0.01	0.02–0.07	5.6
18	Arthritis	48	60	30	21	0.32	0.36–0.37	0.02	0.09–0.11	5.4
19	Arthritis	39	69.1	15	14	0.41	0.45–0.48	0.02	0.04–0.09	5.9
20	Myotonic dystrophy	21	42.3	30	29	0.28	0.25–0.28	0.01	0.01–0.02	6.6
21	Fibrositis	43	64.1	20	14	0.43	0.41–0.44	0.02	0.08–0.19	6.8
22	Myasthenia gravis	24	71	30	10	0.49	0.42	0.02	0.13	7.0
23	Myasthenia gravis	17	48	10–20	34	0.33	0.34–0.36	0.00	0.02–0.08	7.0
24	Chronic nervous exhaustion	53	44.5	15–20	57	0.32	0.28–0.31	0.02	0.03–0.08	7.1
25	Chronic nervous exhaustion	33	40	30	44	0.30	0.28–0.35	0.02	0.03–0.06	7.5
26	Myasthenia gravis	35	55	10	6	0.44	0.38–0.44	0.01	0.01–0.05	7.9
27	Thomsen's disease	29	59.8	30	14	0.51	0.45–0.50	0.01	0.02–0.10	8.5
28	Normal	28	48	20	15	0.42	0.43	0.01	0.04	8.8

One of the important effects of feeding glycine to human subjects is, therefore, an increase in the excretion of creatine. The magnitude of the extra excretion, the time before the initial change occurred, the subsequent rate of increase to a maximum, and the further changes during continued administration of glycine were, on the other hand, quite variable. Differences in these respects were noted especially in the first two groups of subjects, and seemed to be little related to their clinical condition. The variability in the extent of the creatinuria attained after administration of glycine is illustrated by some of the data in table 2. Considering only those subjects who were receiving 30 grams of glycine daily it is seen that although the excretion of creatine before administration of glycine was almost identical, the maximal creatine excretion attained after administration of glycine varied from 0.02 to 0.15 gram nitrogen. This variation is the more striking in light of the fact that the period of observation had been long enough, in the majority of cases represented in table 2 to insure that the maximal creatinuria had been reached.

TABLE 3

Time of occurrence of increased creatine excretion after beginning administration of glycine

	DAY		WEEK					NO CHANGE	TOTAL
	1	2 to 8	2	3	4	7	8		
Miscellaneous and myasthenia gravis									
Males, number	9	7	4	4			1	10	35
Females, number	11	19	6	1	1	1		6	45
Total, number	20	26	10	5	1	1	1	16	80
Per cent	25	33	13	6	1	1	1	20	
Muscular dystrophy									
Males, number	10	4		1					
Females, number	3	2							
Total, number	13	6		1					20
Per cent	65	30		5					

The variability in the time before the initial increase in creatine excretion occurred is illustrated by the data in table 3. It is evident that this increase was observed in groups 1 and 2 any time from the first day to the eighth week after administration of glycine had been begun. The majority were taking 30 grams glycine daily, so that the differences noted cannot be explained as being attributable to the ingestion of varying amounts. An immediate increase occurred in 25 per cent of the subjects,

and within the first week in 58 per cent. In the remaining cases the increase occurred at various later times, as is shown in the table. The creatine excretion of sixteen of the subjects studied remained unchanged. It seems probable that if they could have been observed longer, many of them would have been included in the group of more slowly reacting individuals.

After the initial increase in creatine excretion by a given subject had occurred, the maximum was usually established within a few days. In a few cases, however, the increase was gradual from week to week. Typical

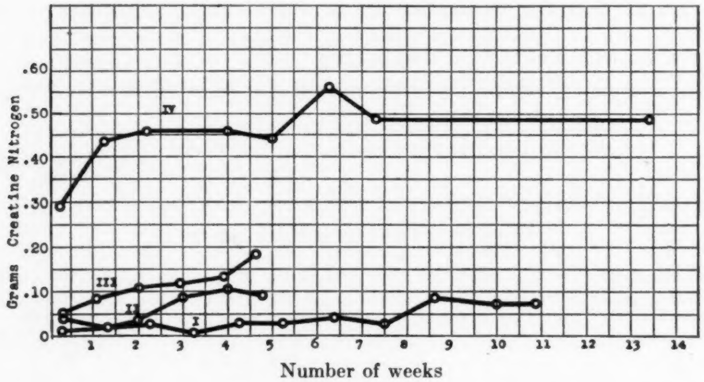

Fig. 1. Curves illustrating various types of changes in the excretion of creatine subsequent to the beginning of administration of glycine.

I. Male, age 37, weight 73.1 kgm. Myasthenia gravis; 7 weeks 30 grams glycine; remaining time 40 grams.

II. Male, age 28, weight 65.9 kgm. Myasthenia gravis; 1 week 20 grams, 2 weeks 30 grams, 2 weeks 40 grams of glycine.

III. Female, age 38, weight 50.5 kgm. Chronic nervous exhaustion; 4 weeks 30 grams glycine.

IV. Male, age 30, weight 89.5 kgm. Muscular dystrophy; 5 weeks 30 grams, 9 weeks 40 grams glycine.

examples of the varied nature of the changes encountered are shown in figure 1. Curve I illustrates the very small fluctuations in the average daily creatine excretion of a subject who had myasthenia gravis, the very slight but questionable tendency toward an increase during the sixth week, and an unmistakable increase during the eighth week. In curve II it is seen that both the initial increase and the maximal creatine excretion occurred sooner than in curve I, and the maximal excretion observed was attained almost simultaneously with the initial rise in the creatinuria. The creatine excretion of the subject represented by curve III, on the other

hand, progressively increased during five weeks of glycine administration. These curves also serve to illustrate a relationship frequently seen, that the creatine excretion of the more slowly reacting individuals usually did not attain as high a maximum.

In marked contrast to the varied behavior of the subjects in groups 1 and 2 in the respects just discussed was the response of those in group 3 to the administration of glycine. As table 3 indicates, the creatine excretion of 95 per cent of the subjects who had progressive muscular dystrophy increased during the first week of administration of glycine. Curve IV in figure 1 illustrates the type of reaction usually observed: the excretion of extra creatine was relatively large, it began almost immediately, and the maximal excretion occurred simultaneously with, or within a few days after, the initial increase.

The majority of all the subjects who had reached a maximal creatine excretion remained at that level as long as the administration of glycine was continued. As has been pointed out, many of the individuals in groups 1 and 2 were slow to attain a maximal excretion. Also the complications accompanying further medication make it necessary to exclude many of the subjects from this discussion. Of thirty-four subjects of groups 1 and 2 who apparently had reached definite equilibrium, twenty-seven maintained approximately their maximal excretion throughout the period of observation. Only seven excreted more creatine during the first interval after administration of glycine had been begun than during subsequent periods, and in no case did the creatine excretion return to the original level. Among the subjects who had progressive muscular dystrophy the maintenance of a high level of creatine excretion was particularly striking. Many of these have been under observation at intervals during six months to two years, and in no case has there been found a subsequent decrease. In this respect our observations fail to confirm those reported by Thomas, Milhorat and Technor (3), and others (6, 7, 8, 9), but are in harmony with the findings of Brand and Harris (4), and Reinhold, Clark, Kingsley and Custer (5).

The question of the influence of the magnitude of the glycine intake on the creatine excretion is, in groups 1 and 2, complicated by the fact that long periods of observation were necessary in many instances to establish a definite equilibrium. In the group of subjects who had progressive muscular dystrophy, however, the response to glycine was rapid and a correlation between the glycine dosage and the extent of the creatinuria could easily be seen. In our cases an increase or decrease in the amount of glycine taken was usually followed by a correspondingly small but definite increase or decrease in the excretion of creatine. This correlation is also illustrated by the data in chart IV in the paper by Harris and Brand (15). A similar correlation was observed for certain of the subjects of groups 1

and 2. After these had come to equilibrium on 20 to 30 grams of glycine, the addition or removal of 10 grams of glycine seemed to cause a slight corresponding change in the creatinuria. However, in several others who had apparently attained a constant rate of creatine excretion on 30 grams of glycine, the addition or removal of 10 grams was without effect.

The influence of glycine on the creatine tolerance. Glycine also appears to influence the creatine tolerance, that is, the ability of the individual to retain ingested creatine. Some results of experiments illustrating this are summarized in table 4. Only those individuals are included in this table who before the administration of glycine were able to retain a large part at least of 1 gram of creatine. It is seen that glycine definitely decreased the ability to retain creatine in practically every case. The extent of this decrease in tolerance and the length of time before any change is demonstrable varied with the individual just as the creatine changes after feeding glycine were seen to vary. The results among subjects who had myasthenia gravis confirm the similar data of Milhorat (16). The table shows, however, that a decrease in creatine tolerance following glycine is not necessarily characteristic of a well defined muscular disease such as myasthenia gravis, but may also occur in other subjects with no definite pathologic disturbance. A series of similar studies on individuals who had a low creatine tolerance before the administration of glycine was also made but the results were much more variable. In some cases the subjects had a still lower tolerance after taking glycine than before, but in several instances there was no appreciable change. In progressive muscular dystrophy Harris and Brand (15) and Cuthbertson and Maclachlan (17) likewise have observed little evidence of a change in creatine tolerance following glycine. Several other investigators have reported that the administration of glycine actually increased the creatine tolerance of these patients, but we have rarely seen such an effect. Many in our series who had been taking glycine for a year or more were still found to excrete practically an entire gram of ingested creatine. On the other hand, unless repeated tests are made, misleading data as to the creatine tolerance may be obtained. For example, the recovery of creatine from one subject who had progressive muscular dystrophy had dropped from 64 per cent before taking glycine to 19 per cent after the subject had been taking glycine for four months. In a test one week later, however, about 90 per cent of the ingested creatine was recovered. Similar fluctuations were observed in the creatine tolerance of some of the subjects in the miscellaneous group. It is possible that differences in dietary conditions may explain these variations.

The inability of patients with progressive muscular dystrophy to retain any appreciable amounts of ingested creatine, together with the fact that administration of glycine results in a markedly increased excretion of

TABLE 4

The influence of glycine on creatine tolerance

SUBJECT	DIAGNOSIS	CREATINE N INTAKE*	GLYCINE GIVEN DAILY, GRAMS	RECOVERY, PER CENT: Before administration of glycine begun	RECOVERY, PER CENT: After administration of glycine begun	NUMBER OF DAYS GLYCINE GIVEN	DATE OF CREATINE TOLERANCE TEST
29	Myasthenia gravis	0.28	0	0		0	11/ 2/33
		0.28	30		8	18	11/22/33
6	Myasthenia gravis	0.28	0	0		0	5/16/33
		0.28	20–30		71	11	5/29/33
		0.28	20–40		71	35	6/23/33
30	Effort syndrome	0.28	0	0		0	3/21/33
		0.28	30		39	20	4/13/33
31	Sciatic syndrome	0.28	0	3.2		0	10/14/33
		0.28	15		0	14	10/31/33
		0.28	15		17	55	12/11/33
14	Chronic nervous exhaustion	0.28	0	4		0	6/23/33
		0.28	20		0	14	7/10/33
32	General fatigue	0.28	0	5		0	10/31/33
		0.28	30		37	70	12/13/33
10	Myasthenia gravis	0.28	0	7.1		0	1/ 1/33
		0.28	30		23	11	1/16/33
		0.28	30–35		36	112	4/20/33
33	Progressive muscular dystrophy	0.28	0	28		0	4/12/33
		0.28	30		71	17	5/ 2/33
		0.28	30–40		51	45	5/29/33
18	Arthritis	0.28	0	32		0	10/19/33
		0.28	30		46	16	11/ 6/33
13	Myasthenia gravis	0.28	0	32		0	4/10/34
		0.28			77	11	4/23/34
34	Chronic nervous exhaustion	0.28	0	39		0	9/12/33
		0.28	30		105	26	10/11/33

* This is in addition to the creatine of the diet.

creatine, indicates that the glycine causes an additional formation of creatine, rather than merely a decreased retention of food creatine or a forcing out of creatine normally present in the tissues. In several subjects who were observed daily for periods of two to four months, the extra creatine excreted during the administration of glycine was equivalent to 25 to 50 per cent of their total creatine stores. Such losses of muscle creatine would undoubtedly be reflected in the clinical condition of the subjects, but in reality their condition remained practically unchanged. The excretion of extra creatine by subjects who have been observed at intervals throughout one to two years has far exceeded the calculated body creatine. In one instance 261 grams of extra creatine were lost during two years of administration of glycine, whereas the calculated creatine stores of the subject amounted to only about 37 grams. These estimates of extra creatine incident to administration of glycine were arrived at on the assumptions that the levels of excretion of creatine, as determined before administration of glycine, did not materially change, and that the average excretion of creatine during the periods of administration of glycine remained reasonably constant. Both of these assumptions, we believe, are amply justified not only by our data, but also by the data of Harris and Brand.

The influence of glycine on the excretion of creatinine. The average creatinine excretion of the majority of subjects, from week to week, was remarkably constant in spite of the fact that the diets taken were not creatine-creatinine free. The daily fluctuations were somewhat greater than the weekly fluctuations, particularly in certain individuals. In most instances, however, the daily excretion of creatinine was approximately constant and in no case were the fluctuations comparable in magnitude to many of those currently reported in the literature. It seems that many of the data offered on this subject may be complicated by grossly inaccurate collections of the twenty-four hour urine specimens. The administration of glycine usually has not been followed by changes in the creatinine elimination. When changes were encountered they were neither large nor abrupt, but always slow and gradual. The extreme variations in the creatinine excretion observed for one group of individuals is illustrated by data in table 2, while table 5 shows more detailed data for three subjects. The very constant creatinine elimination of subject 35 before and during administration of glycine is typical of that observed in the majority of the cases studied. In all, sixty-two subjects exhibited this constant creatinine excretion. Many were not under observation as long as subject 35, however, and it is possible that definite changes might have occurred in a longer period. In these sixty-two are included some of those in groups 1 and 2 and all of those who had progressive muscular dystrophy. Although many of the latter were under observation a year

or more, in no case was the creatinine excretion materially different after administration of glycine than before. This again confirms the findings of Brand and Harris (4) in progressive muscular dystrophy. The gradually but definitely increasing creatinine excretion such as that illustrated by subject 1 was seen in only five subjects. In twenty-two other cases there were only slight increases, but in many of these the period of observation was less than one month. The definitely decreasing values seen for subject 36 in table 5 were also found in three other cases, in all of which, however, the subjects were in very serious condition and were becoming rapidly worse in spite of any type of treatment. The excretion of creatinine by three other subjects was decreased, but the decrease was small compared to that shown for subject 36. It is impossible at present to

TABLE 5

The excretion of creatinine before and after the administration of glycine

SUBJECT	GLYCINE GIVEN DAILY, GRAMS	AVERAGE DAILY										
		Preformed creatinine nitrogen before administration of glycine begun, grams	Preformed creatinine nitrogen after administration of glycine begun, grams									
35	30	(4) 0.38	(14) 0.39	(8) 0.38	(6) 0.39	(10) 0.40	(7) 0.41	(6) 0.42	(10) 0.40	(6)* 0.39		
1	30	(2) 0.49	(7) 0.47	(7) 0.53	(16) 0.55	(7) 0.59	(7) 0.61	(12) 0.63				
36	30	(4) 0.53	(7) 0.54	(7) 0.51	(21) 0.52	(9) 0.49	(11) 0.46	(6) 0.43	(5) 0.42	(14) 0.41	(8) 0.40	(30) 0.39

* The numbers in parentheses indicate the number of days in each period, and are for consecutive periods except for this one figure. This was obtained after an interval of one year of feeding glycine at an average dose of 30 grams daily.

draw any definite conclusions from these results, but it is apparent that under many conditions glycine exerts little influence on the excretion of creatinine. Further extended investigations with subjects on controlled diets are necessary to determine whether the changes observed in some cases are attributable to glycine or to other complicating factors.

The influence of glutamic acid on the excretion of creatine. The question as to whether the ability of glycine to influence the excretion of creatine is a property of amino-acids in general has been considered by several investigators. Our experiments with glutamic acid are less extensive than those with glycine; nevertheless, sufficient data have been obtained to warrant a brief description of them. Eleven subjects who had progressive muscular dystrophy, and one normal child, have been fed

glutamic acid or sodium glutamate under various conditions. The addition of glutamic acid caused no marked change in the creatine excretion of six of the subjects who were already receiving glycine. Three children of the same family, one of whom was normal, were fed daily 10 to 15 grams of sodium glutamate with a subsequent slight reduction in creatine excretion. Two other children were found to excrete the same amount of creatine after they had taken 10 to 20 grams of sodium glutamate, as they excreted before, whereas the feeding of 15 grams of glycine to one of these children caused an immediate increase in the excretion of creatine nitrogen from 0.05 to 0.11 gram. Conversely, in two other subjects who were receiving both glycine and glutamic acid, the withdrawal of glycine resulted in an immediate decrease of the creatine excretion to or below the original level prevailing before either amino-acid was fed. An immediate return to the higher level occurred with the restoration of glycine. Although these investigations have been for relatively short periods of time the group of subjects considered here are those whom we have already shown to react to glycine immediately. In only one case was there any evidence that the feeding of glutamic acid may have caused an increase in the excretion of creatine, and this was slight. The majority of results with glutamic acid, therefore, are in marked contrast to those with glycine. The amounts of glutamic acid given were similar to those used by Tripoli and Beard (6). We have failed, however, to note any such marked changes as they have reported but have confirmed Brand, Harris, Sandberg and Ringer (2), Thomas, Milhorat, and Technor (3), and Allinson, Henstell, and Himwich (18) who also failed to find an increased creatine excretion on the administration of glutamic acid.

Discussion. Evidence of the possibility of influencing the creatine metabolism by administration of glycine as judged by the results of the analytic methods employed, is seen in the increased excretion of creatine and in the decreased creatine tolerance following its administration. Harris and Brand (15) showed that the removal of glycine from the body by the daily ingestion of benzoic acid leads to a prompt decrease in the creatine excretion of subjects who had progressive muscular dystrophy, an observation which they offered as further evidence of the importance of glycine in the formation of creatine. Linneweh and Linneweh (19), on the other hand, stated, without presenting data, that they had failed to confirm Harris and Brand (15) in this respect. Shorr, Richardson and Wolff (20) reported that they frequently had observed a significant increase in creatine excretion on the day benzoic acid had been given. The data presented by all of these investigators is insufficient to determine the reasons for these differences.

It is possible that glycine itself may be a precursor of creatine or it may merely stimulate or change the course of the metabolism in general.

None of the data so far reported give any definite information as to the mechanism involved. Benedict and Osterberg (21) have suggested that the creatinuria reported by some investigators as the result of high protein feeding is attributable to a forcing out of small quantities of creatine from the tissues as the result of the influx of other nitrogenous compounds. In the case of glycine, however, it appears that there is a definitely increased formation (or decreased destruction) of creatine rather than a forcing out of normally preëxistent creatine. The evidence is still insufficient to determine whether the increased excretion of creatine following administration of glycine is peculiar to this amino-acid. The difference in the results produced by feeding glycine and glutamic acid have been pointed out. In view of the effect of glycine on the creatine excretion it is also interesting to consider the studies of Denis and her associates (22, 23, 24), on the influence of protein diets on normal women, normal children, and patients who had hyperthyroid diseases. The excretion of creatine by all of these was found to be greater when they were receiving a high rather than a low protein diet, and the maximal excretion was obtained when the diets included gelatin, a protein which contains a high percentage of glycine. When similar high protein diets containing gelatin were given to two normal male subjects for twelve to fourteen days no creatinuria was produced.

Numerous other investigations of the effect of feeding high protein diets and the individual amino-acids, particularly arginine, on the creatin-creatinine metabolism have been carried out. The results, especially of the early investigations, have been most varied. Some of the more recent studies indicate that the variations may be attributable to several factors. The slow increase in the excretion of creatinine when creatine is fed daily to normal dogs, as reported by Benedict and Osterberg (21), and confirmed for normal human subjects by Chanutin (25), and by Rose, Ellis and Helming (26), and the frequently slow change in the excretion of creatine and in the creatine tolerance produced by glycine which we have observed, all indicate the importance of the time factor in such studies. That the protein level is also of great importance is indicated by the experiments of Bollman (27) who could observe in dogs practically no evidence of the conversion of ingested creatine to creatinine during nine weeks of administration of a low protein, meat-free diet, but who did obtain evidence of such conversion within two weeks, when the diet was supplemented with 100 grams of casein.

The use of essentially creatine-free diets in investigations of the metabolism of creatine and creatinine is another factor of obviously great importance. This is particularly true, for example, in the interpretation of the influence of glycine on the creatine excretion by the group of individuals who have a normally high creatine tolerance. Unless such sub-

jects are receiving, on the average, their usual normal amounts of creatine it may be difficult or impossible to obtain evidence of excretion of extra creatine when glycine is given. Whether or not the use of creatine-free diets in studies of subjects whose creatine tolerance is greatly reduced, such as patients who have progressive muscular dystrophy, is of great importance, is not clear. It is evident that the contradictory results which have been reported for this group of subjects cannot be explained entirely on this basis. During our own studies of such subjects they took their customary diets. Nevertheless, the results generally have confirmed those obtained by Harris and Brand (15) on a similar group of subjects who were taking creatine-free diets. On the other hand, we have failed to confirm the results of Tripoli and Beard (6), although it appears that these investigators, also, did not restrict their subjects to creatine-free diets.

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THE EFFECT OF DENERVATION ON THE SENSITIVITY TO ADRENINE OF THE SMOOTH MUSCLE IN THE NICTITATING MEMBRANE OF THE CAT

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The increased sensitivity of smooth muscle to adrenine after destruction of its sympathetic nerve supply was first observed by Meltzer and Auer (1904) in the rabbit's pupil. They noted that the phenomenon did not appear earlier than 24 hours after removal of the superior cervical ganglion and that postganglionic section was more effective than preganglionic decentralization in the development of the augmented sensitivity. Similar experiments on cats (Meltzer, 1904) yielded like results; sensitization of pupil and nictitating membrane to subcutaneous injection of adrenine was seen only when the superior cervical ganglion had been removed at least 48 hours previously. Elliott (1905) confirmed and extended these observations to other smooth muscle structures innervated by the sympathetic nervous system.

Much use has been made of denervated smooth muscle for the detection and quantitation of adrenine (Elliott, 1912; Kellaway, 1919; Hartman, McCordock and Loder, 1923; Rosenblueth, 1932b; Freeman, Smithwick and White, 1934), for the detection of sympathin (Cannon and Bacq, 1931; Rosenblueth and Cannon, 1932), and for studying the action of drugs (Dale and Laidlaw, 1912; Rosenblueth, 1932a). In spite of this widespread use of sensitized smooth muscle definite information concerning the time required for complete development of sensitization is for the most part lacking; various investigators have allowed intervals ranging from a few days to several weeks for sensitization to take place in their preparations. Quantitative determinations of adrenine sensitivity in isolated structures at various intervals after denervation are not comparable, since such experiments cannot be carried on in a single animal (see Shimidzu, 1924). In order to follow the course of sensitization, a structure must be chosen, the responses of which may easily be determined at frequent intervals during the process.

The nictitating membrane of the cat offers many advantages for the study of this phenomenon. Its contractions may be recorded in situ, and it therefore provides an excellent chronic preparation. The smooth muscle

exerts considerable force in its response (Hampel, 1934), and so a high degree of magnification may be used in the recording system to detect and register very slight responses. There is, moreover, very little tendency for the muscle to display rhythmic contractions in the anesthetized preparation (Rosenblueth and Bard, 1932). In both isotonic and isometric contractions it shows graded responses to graded doses of adrenine introduced into the circulating blood (Rosenblueth, 1932b). Finally, the muscle is innervated solely by the cervical sympathetic (Rosenblueth and Bard, *loc. cit.*), which innervation allows of either decentralization or denervation (Bishop and Heinbecker, 1932; Cleveland, 1932). The study of the process of sensitization in the nictitating membrane after interruption of its sympathetic supply has been further prompted by its use as an indicator of sympathetic activity (Rosenblueth and Cannon, 1932; Rosenblueth, 1932b and c).

METHOD. Anesthesia produced by nembutal (0.7 cc. per kgm. intraperitoneally) was found satisfactory for both operative and experimental procedures. At the conclusion of an experiment an intraperitoneal injection of 50 cc. of normal salt solution was given to shorten the remaining period of anesthesia.

Denervation of the nictitating membrane (n.m.) was accomplished by removal of the superior cervical ganglion (sup. cerv. gang.); decentralization involved only section of the cervical sympathetic nerve (cerv. sym. n.) and removal of a short piece to prevent immediate regrowth. In most cases one n.m. was denervated and the other decentralized at the initial operation. The isotonic or isometric responses of the membranes to a series of graded doses of adrenine injected into the femoral vein at a fixed rate were recorded immediately after the operation and at subsequent intervals of two or three days thereafter until no further change in magnitude of response was obtained. The remaining sup. cerv. gang. was then removed and records again taken until the corresponding n.m. reached maximal sensitivity.

The methods for recording contractions of the n.m. previously described (1934) were adapted to permit simultaneous registration from both membranes. Isotonic responses were recorded by means of two writing levers, each having a 15-fold magnification and exerting a load of about 7 grams on the membrane. In the series of experiments in which isometric responses were measured, two spring levers having approximately an 8-fold magnification and allowing 1 cm. excursion for each 5 grams tension-increment were used. A uniform initial tension of 5 grams was imposed upon each n.m. in these tests.

The adrenine solutions injected were made up from freshly prepared stock solutions of adrenalin (Parke, Davis & Co.) 1:100,000 or 1:50,000. The injection volume was in all cases the same (1 cc.) and was introduced at a uniform rate over a period of 10 seconds.

Results. *Isotonic responses to standard injections of adrenine after denervation.* A comparison of the effects of preganglionic and postganglionic denervation on the isotonic responses of the n.m. to the intravenous injection of a series of standard doses of adrenine in successive experiments covering a period of 28 days was obtained in cat 3. In this animal the right cerv. sym. n. was sectioned, and the left sup. cerv. gang. was removed on January 6, 1934. Immediately after the operation the isotonic responses evoked by the injection of 0.25, 0.50, 0.75 and 1.0 cc. of adrenine,

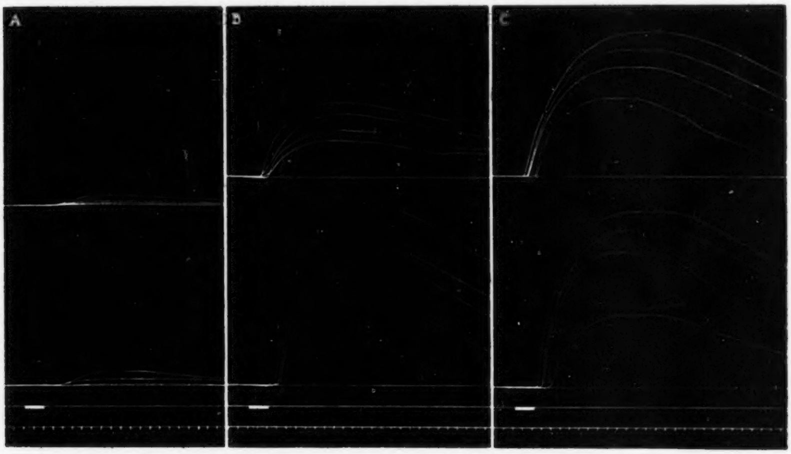

Fig. 1. Cat 3. Isotonic responses of the right n.m. (above) and left n.m. (below) to intravenous injections of 0.25, 0.50, 0.75 and 1.0 cc. of adrenine, 1:100,000. Injections during 10 seconds at signal. Time in 5-second intervals.

A. 1/6/34. Immediately after section of right cerv. sym. n. and removal of left sup. cerv. gang.

B. 1/20/34. Responses recorded 14 days after first operation and immediately after removal of right sup. cerv. gang.

C. 2/3/34. Responses recorded 28 days after initial operation; 14 days after second operation.

1:100,000 were recorded (fig. 1A). Similar experiments were performed 2, 5, 7, 10 and 12 days after the operation. On the fourteenth day the decentralized right sup. cerv. gang. was excised and records again taken immediately thereafter (fig. 1B). Further determinations were made on this animal 16, 18, 21, 24, 26 and 28 days from the beginning of the series. The last of these records is shown in figure 1C. Measurements of the heights of contraction taken from the original records are to be found in table 1, together with a record of the body-weight changes in the animal during the period of experimentation. The responses of the two mem-

branes to the injection of 1 cc. of adrenine, 1:100,000, are shown graphically in figure 2, which reveals a striking increase in magnitude of response of the left n.m. during the first few days following denervation and then a more gradual increase until a maximum is reached. In the right n.m., which was decentralized first and completely denervated 14 days later, the increase in response takes place in two stages. Curves representing responses to the other adrenine doses used are remarkably similar.

A like series of experiments was performed on cat 1. The right sup. cerv. gang. was excised, and the left cerv. sym. n. was sectioned at the first operation. The left ganglion was taken out 13 days later. The data

TABLE 1

Cat 3. Responses (cm.) to adrenine (1:100,000)

DATE	BODY WEIGHT	RIGHT NICTITATING MEMBRANE				LEFT NICTITATING MEMBRANE			
		0.25 cc.	0.50 cc.	0.75 cc.	1.00 cc.	0.25 cc.	0.50 cc.	0.75 cc.	1.00 cc.
	kgm.								
1/ 6/34*	2.0	0.08	0.16	0.25	0.50	0.00	0.05	0.30	0.69
1/ 8/34	1.9	0.21	0.85	1.54	1.90	1.75	3.40	4.65	4.80
1/11/34	2.0	0.72	1.57	2.28	2.70	2.52	4.57	6.03	6.37
1/13/34	1.9	0.84	2.05	2.68	3.13	3.17	5.58	6.82	7.55
1/16/34	1.9	1.65	2.00	3.00		3.75	5.70	6.80	
1/18/34	1.8	1.60	2.22	2.73	3.50	3.09	5.45	6.15	7.15
1/20/34†	1.8	1.70	2.15	2.95	3.50	4.38	5.53	6.28	8.07
1/22/34	1.8	3.20	4.35	5.25	5.80	3.70	5.30	6.10	7.30
1/24/34	1.8	4.02	5.60	6.35	6.75	4.30	6.09	7.05	7.85
1/27/34	1.8	3.25	4.72	5.46	6.55	4.25	5.72	6.86	7.93
1/30/34	1.8	2.56	4.45	5.60	6.15	4.20	5.88	7.45	8.17
2/ 1/34	1.8	3.83	5.75	6.55	7.20	4.20	6.15	7.48	7.96
2/ 3/34	1.8	3.68	5.15	5.92	6.75	3.40	6.20	7.22	8.02

* Right cerv. sym. n. cut and left sup. cerv. gang. removed before the experiment.
† Right sup. cerv. gang. removed before the experiment.

obtained in 11 experiments on this animal over a period of 24 days are presented graphically in figure 3. The greatest variation in body weight in this animal during the experimental period was 5 per cent from the initial weight.

The results on three additional cats were the same as those reported above. In one of these animals a determination made 77 days after the initial operation showed no greater response than on the 24th day of the series.

It will be noted that the left n.m. appeared to reach a higher level of responsiveness than the right n.m. (see figs. 2 and 3). This difference was found to be due to a slight difference in the recording levers. Although the magnification afforded by the two levers was identical, the lever used

for recording responses of the right n.m. imposed a slightly greater load (about 1 gm.) on that structure. The effect of slight changes in load on the isotonic response of the n.m. has already been reported (Hampel, 1934).

Isometric responses to standard injections of adrenine after denervation. The relationship between the amount of adrenine injected and the response of the smooth muscle of the n.m. is essentially the same for isotonic

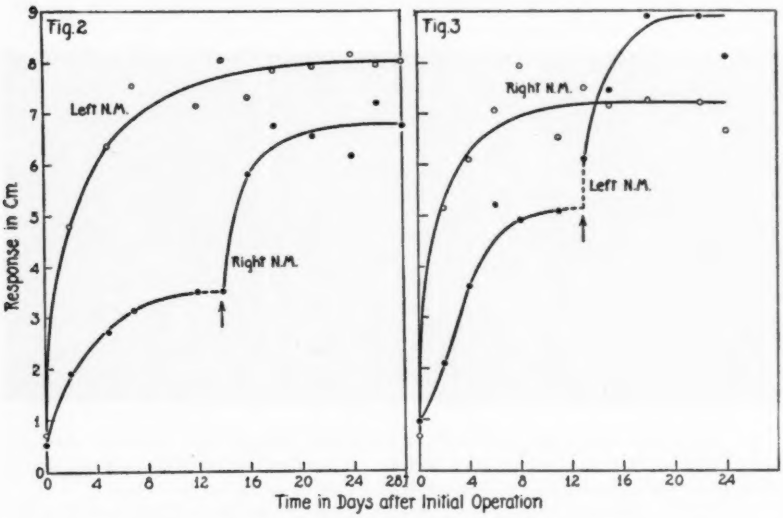

Fig. 2. From data in table 1. Isotonic responses of the nictitating membranes to 1 cc. of adrenine, 1:100,000, after removal of the left sup. cerv. gang. and section of the right cerv. sym. n. The right sup. cerv. gang. was removed 14 days after section of the right nerve, as indicated by the arrow.

Fig. 3. Cat 1. Isotonic responses of the nictitating membranes to 1 cc. of adrenine, 1:100,000, after removal of the right sup. cerv. gang. and section of the left cerv. sym. n. The left sup. cerv. gang. was removed 13 days after section of the left nerve, as indicated by the arrow.

and isometric methods of recording (Rosenblueth, 1932b). It was therefore of interest to attempt a confirmation of the above results by measuring the tension developed by the n.m. in response to standard doses of adrenine in successive experiments after denervation. The procedure was essentially the same as in the foregoing experiments. In order to be certain that the initial stress acting on the membrane was the same in all tests, an initial tension of 5 grams was applied to the membrane for at least 5 minutes before starting each determination.

A complete series of tests was performed on cat 7. In this animal the left n.m. was denervated by removal of the left sup. cerv. gang. and the right membrane decentralized by section of the right cerv. sym. n. at the initial operation. The isometric responses to 0.25, 0.50 and 1.0 cc. of adrenine, 1:50,000, were recorded immediately after the operation (fig. 4A) and 1, 3, 6, 10 and 14 days thereafter. When the experiment on the 14th day of the series (fig. 4B) had been completed the right sup. cerv. gang. was removed and further tests performed 15, 17, 20 and 24 days (fig. 4C) after the initial operation. In figure 5 the responses to 1.0 cc.

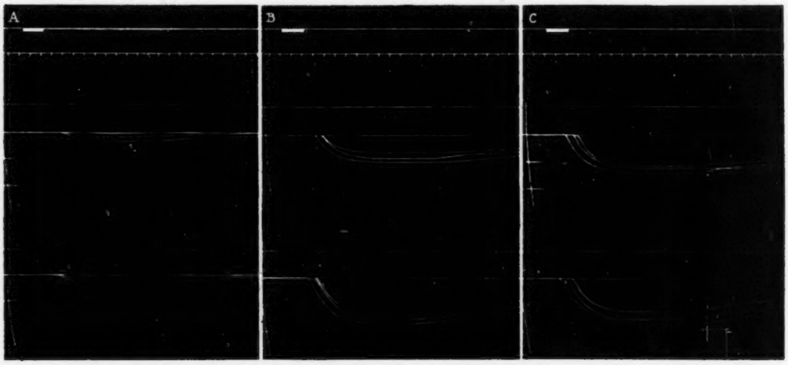

Fig. 4. Cat 7. Isometric responses of right n.m. (above) and left n.m. (below) to 0.25, 0.50 and 1.0 cc. of adrenine, 1:50,000. Injections during 10 seconds at signal. Time in 5-second intervals. Calibration of levers in 5 grams.

A. 2/23/34. Responses recorded immediately after section of right cerv. sym. n. and removal of left sup. cerv. gang.

B. 3/9/34. Responses recorded 14 days after operation. Immediately after the experiment the right sup. cerv. gang. was removed.

C. 3/19/34. Responses recorded 24 days after initial operation; 10 days after second operation.

of adrenine, 1:50,000, in the successive experiments are represented graphically. The curves are essentially the same as those relating the isotonic responses reported above. This animal was in excellent condition throughout the experimental period and showed a slight increase in weight (6 per cent) during that time.

A series of 5 experiments during 14 days was made on cat 8. Again, the left sup. cerv. gang. was removed and the right cerv. sym. n. was cut at the same operation. Records of the isometric responses to the different doses of adrenine were made on that day and 3, 6, 10 and 14 days thereafter. In figure 6 are plotted the curves for the increase in response of the two membranes to the 1 cc. injections of adrenine, 1:50,000. The

animal died during an attempt to remove the decentralized right ganglion after the last experiment.

Another cat which provided 9 experiments in 21 days gave similar results.

DISCUSSION. The terms "increased sensitivity" and "sensitization" have been used by various authors to describe the condition of increased responsiveness that is found to exist after denervation. If sensitivity is to be defined as the reciprocal of the dose required to produce any given

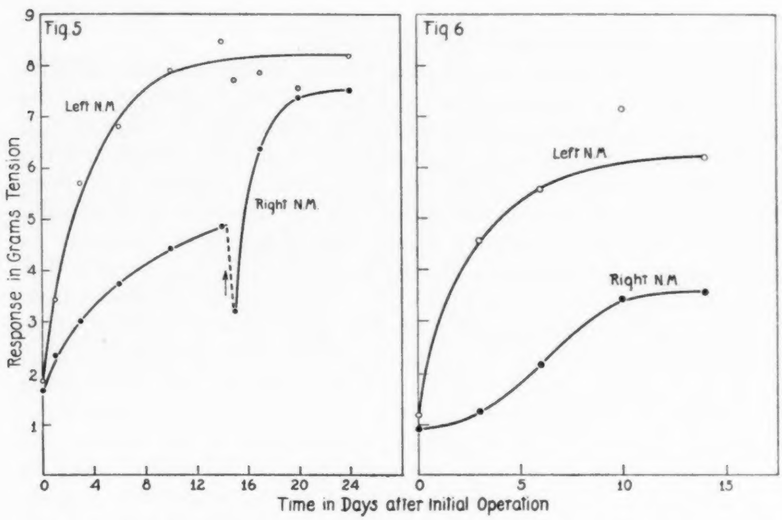

Fig. 5. Cat 7. Isometric responses of the nictitating membranes to 1 cc. of adrenine, 1:50,000, after removal of the left sup. cerv. gang. and section of the right cerv. sym. n. The right sup. cerv. gang. was removed 14 days after section of the right nerve as indicated by the arrow.

Fig. 6. Cat 8. Isometric responses of the nictitating membranes to 1 cc. of adrenine, 1:50,000, after removal of the left sup. cerv. gang. and section of the right cerv. sym. n.

response, then the results reported above may be considered as indicative of an increased sensitivity of the smooth muscle to adrenine, and the curves relating the increase in response to a standard dose of adrenine after denervation may be taken as indicating the course of increased sensitivity.

The reason for using a series of standard doses of adrenine in these experiments is quite obvious. The use of a single standard dose throughout the tests might well be objected to on the grounds that such a dose might be practically maximal at the level of greatest sensitivity of the

muscle. In such a case, the time required for development of maximal sensitivity would have been less than the actual value. It was therefore considered advisable to employ a series of standard doses, the responses to which might be used as a check on one another.

On the basis of the results obtained, it is clear that in the use of denervated smooth muscle for the detection and estimation of adrenine and adrenine-like substances, the most sensitive preparation is obtained by denervation 14 to 15 days previously. The preparation and methods used in these experiments might well be adapted to the quantitative determination of adrenine and similar substances. As these tests indicate, the chronic preparation can be used repeatedly.

No definite explanation is evident at present for the increased sensitivity which occurs in smooth muscle as a result of denervation, nor do the results reported here warrant any statement concerning the cause of such sensitization. It is, however, interesting to consider the theories offered by previous authors to explain the phenomenon. Cannon and Bacq (1931) used denervated smooth muscle for the detection of sympathin and found that the denervated organs showed a greater sensitivity to sympathin as well as adrenine. They stated that "it is quite possible that the rising sensitiveness of an organ after being denervated results from accumulation of sympathin within the idle cells—when touched off, there is more sympathin present to act in coöperation with adrenin." Later, one of them (Bacq, 1933) found this view no longer tentable on the basis of results he obtained from the action of complex sympathomimetic polyphenols on denervated smooth muscle, and proposed a new theory. He assumes that after denervation the production of sympathin ceases in cells which are normally under a constant sympathetic tonus (muscle cells of heart, iris, and nictitating membrane), and there occurs a detoxication of the cells, in consequence of which a lowered threshold to sympathomimetic polyphenols obtains. He points out that the relationship between the degree of sympathetic tonus before denervation and the degree of hypersensitivity to adrenine after denervation (see Elliott, 1905) can be explained on this hypothesis. Bacq's theory is in many ways attractive, and the defensive position of such an hypothesis is readily recognized.

Another and quite different theory is that offered by Rosenblueth (see Rosenblueth and Morison, 1934), which postulates that an increase in permeability of the cells takes place as a result of denervation, or upon the administration of cocaine. There is considerable evidence from the literature to support this theory. Some workers have found a change in the permeability of cells after sympathetic denervation. Alpern (1928) reported that the per cent of sodium chloride in the saliva from the dog's submaxillary gland was increased after removal of the superior cervical

ganglion. The excretion of injected urea by the gland was also increased under the same conditions. He produced evidence to show that other glands of the body likewise exhibit an augmented permeability after the influence of the sympathetic nervous system has been removed. Gabbe (1926) showed that the permeability of the capillaries of striped muscle was increased after sympathetic denervation in guinea pigs, as evidenced by intravenous staining methods and the speed of the dehydration process after intravenous injection of 10 per cent sodium chloride solution. He was unable to demonstrate any change in the permeability of striped muscle cells themselves.

The theory that an increased permeability of the smooth muscle cells accounts for the increased sensitivity to sympathomimetic substances after denervation could also be brought forth to explain a similar increase in sensitivity to substances which are generally not sympathomimetic. Of particular significance in this regard is the work of Rosenblueth (1932a) on the effect of various drugs on the nictitating membrane. He found that the contractions of the smooth muscle of the membrane in response to acetylcholine, pilocarpine, physostigmine, and histamine were, in each case, to some extent increased by previous denervation. Attention may also be directed to the results of Dale and Laidlaw (1912) who, in studying the effects of certain nicotine alkaloids on the smooth muscle of the cat's eye, observed an abnormal sensitiveness to the peripheral action of these agents after degeneration of the postganglionic sympathetic fibers. More recently Bacq and Rosenblueth (1934) found that intravenous injections of calcium chloride and potassium chloride alike produced contraction of the nictitating membrane of the cat, and further observed that the responses were greater after degeneration of the nerve supply to the muscle.

If an increased permeability of the smooth muscle is to account for the augmented sensitivity to various substances acting on the muscle, it is evident that such increased permeability would also increase the outward diffusion of substances produced in the smooth muscle. We should expect, then, that such a substance, sympathin, which is produced in smooth muscle by stimulation of its sympathetic nerve supply (Cannon and Bacq, 1931), would be liberated with greater ease under conditions of increased permeability. Proof on this point is unfortunately not to be looked for in the case of sensitization after denervation, since the degeneration of the nerve supply precludes the possibility of evoking sympathin production. Rosenblueth and Morison, however, have recently (1934) been successful in demonstrating that the output of sympathin is facilitated by the administration of cocaine, which produces a sensitization similar to that resulting from degeneration of the sympathetic nerve supply (see Rosenblueth, 1932a). They have attributed the augmented output of sympathin by the heart upon stimulation of the accelerator nerves under

the influence of cocaine (as evidenced by responses of the nictitating membrane and controlled by adrenine injections), to an increased permeability of the tissues where sympathin is elaborated. The possibility, therefore, that sensitization resulting from denervation is the expression of an increased permeability is not too remote. It appears plausible enough to be used as a working hypothesis in future research on the problem.

SUMMARY

The isotonic and isometric responses of the smooth muscle of the cat's nictitating membrane to standard doses of adrenine injected intravenously have been recorded immediately after sympathetic denervation and at frequent intervals thereafter (every two or three days) until no further change was observed (see figs. 1 and 4).

During the first 6 to 8 days after removal of the superior cervical ganglion the magnitude of response of the corresponding nictitating membrane to the same dose of adrenine rapidly increases. A more gradual increase takes place during the succeeding 6 to 8 days, after which further change is slight (see figs. 2, 3, 5 and 6).

The increase of response as a result of decentralization (section of the cervical sympathetic nerve) bears the same time relations, but the extent of the increase is approximately half as great as after denervation (figs. 2, 3, 5 and 6). After removal of the decentralized ganglion a second increase of response occurs, and the final level of responsiveness attained is about equal to that obtained by denervation without previous decentralization (figs. 2, 3 and 5).

The definition of sensitivity as the reciprocal of the dose required to produce a given response allows the results reported to be regarded as a measure of increasing sensitization to adrenine after denervation of the smooth muscle investigated.

The various theories advanced by previous authors to explain the phenomenon of sensitization after denervation are discussed. The recent hypothesis of Rosenblueth that a change in permeability occurs in smooth muscle as a result of denervation is considered the most satisfactory explanation of the phenomena observed.

I wish to express my thanks to Dr. W. B. Cannon for his interest and help in this work and to Dr. A. Rosenblueth for valuable suggestions.

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CERTAIN BLOOD CHANGES ASSOCIATED WITH PHYSICAL EXHAUSTION IN THE NORMAL DOG[1]

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The purpose of the work to be presented here is to describe the changes in certain blood constituents resulting from exercise carried to exhaustion. It comprises an analysis of the results of 46 experiments on 6 dogs, one exercised at room temperature on an inclined treadmill, the others by swimming in water maintained at a temperature of 38°C.

Rice and Steinhaus (1) have shown that in dogs exercised on a treadmill with a 22 per cent grade there occurred a reduction in the bicarbonate concentration and CO_2 tension of the blood serum and a marked rise in pH, results which were explained as an effect of over-ventilation, since heating the dogs without exercise led to similar blood changes. When dogs were exercised by swimming in water at 15°, there occurred a fall in serum bicarbonate, a rise in CO_2 tension, and a fall in pH, results somewhat similar to those observed in man exercising on a bicycle ergometer. Swimming in water at 40° gave results similar to treadmill exercise and overheating, while swimming at 30° resulted in only very slight acid-base changes.

One of us (2) found in 1921 that in the initial stages of exercise, the blood lactic acid rose markedly, but with continued exercise declined to normal values. This fact has been thoroughly studied more recently by Campos, Cannon, Lundin and Walker (3). They found that blood lactic acid rose in the dog during the first stages of work on an inclined treadmill, then declined. There was no close relation between lactic acid in the blood and the performance of the animal. They further found that the blood sugar usually fell at exhaustion but that exhaustion could occur when the glycemic level was high. There was no close relation between levels of glucose and lactic acid in the blood.

Dische and Goldhammer (4) found that long continued running on the

[1] This work has been conducted under a grant from the Douglas Smith Foundation at the University of Chicago.

[2] The authors are deeply indebted to Dr. Grover Hulla and Miss Helen Oldham for valuable assistance in the experimental work.

treadmill led to a pronounced fall in blood sugar and inorganic phosphorus but to an increase in acid-soluble phosphorus.

EXPERIMENTAL PROCEDURE. Six short-haired male dogs weighing 6 to 13 kgm. were used for the experiments. In each experiment they were exercised to exhaustion, five by swimming in a tank of water at 38°C., one, dog 1, by running at a speed of 165 meters per hour on a treadmill with a 20 per cent incline. In the latter case, the room temperature varied from 22.5° to 28.5°C., and the dog's rectal temperature varied during exercise from 39.5° to 41°C. Rectal temperatures for the other dogs while swimming varied from 38° to 40.5°. Venous blood samples were drawn immediately before exercise, at 15, 60, 150, and 210 minutes after exercise was begun, at exhaustion, and after 30 and 120 minutes of rest following exercise. Usually the exercise was interrupted only 3 to 5 minutes for drawing blood samples and recording the dog's temperature.

TABLE 1

DOG NUMBER	TYPE OF EXERCISE	NUMBER OF EXPERIMENTS	TIME TO EXHAUSTION		
			Minimum	Maximum	Average
			minutes	*minutes*	*minutes*
1	Treadmill	13	84	372	165
2	Swimming	5	83	217	136
3	Swimming	7	84	422	174
4	Swimming	6	150	297	204
5	Swimming	9	91	283	214
6	Swimming	6	59	232	130

A total of about 100 to 200 cc. of blood was drawn during an experiment. The experiment was usually repeated at weekly intervals. Routine hematocrit determinations showed that cell volume gradually declined from 45 to 30 per cent during the series of experiments, but the difference from one experiment to the next was very small. The number of experiments on each dog, the type of exercise, and the time elapsed before exhaustion are shown in table 1.

The following analyses were made on the blood samples: cell volume by the Van Allen hematocrit (5), serum pH by the colorimetric method of Hastings and Sendroy[3] (6), total serum CO_2 by the Van Slyke and Neill manometric blood gas technique (7), serum lactate by the gasometric method of Avery and Hastings (8), and in many instances by the Friedemann-Shaffer (9) method as well, and serum sugar by the micro method

[3] The serum pH values were read at 38°C. and recorded without further correction. This means that they are approximately 0.1 pH higher than the electrometric pH of the undiluted serum at the same temperature.

of Folin (10). Serum bicarbonate and CO_2 tension were calculated from total CO_2 and pH values.

Experimental results. *Acid-base balance.* The results for a given dog, exercised under constant conditions as far as we were able to regulate them, showed considerable variation in the degree of change produced, although qualitatively the changes were consistent from experiment to experiment. The curves in figure 1 were obtained by averaging the results for each dog and plotting them against minutes of exercise. They represent what might be called the average response.

The serum lactate concentration rose and the bicarbonate concentration fell markedly within the first 15 minutes of exercise; with continued exercise these tended to return toward the initial values; at exhaustion, however, a second rise in lactate and a fall in bicarbonate frequently occurred. With rest there was a partial recovery within a half hour and a complete return of both bicarbonate and lactate to the initial concentrations at the end of two hours. The curves of the bicarbonate changes appear to be approximate mirror images of the lactate curves. In general, the same type of change with exercise was shown by all of the dogs, whether exercise consisted in swimming or running on a treadmill. Attention might be called to the experiments on dog 6, in which the lactate concentration subsequent to the 15 minute exercise period fell almost to the initial level and remained at that low level even at exhaustion. It is particularly significant that this dog showed a marked hypoglycemia even before the onset of exhaustion.

Serum pH changes with exercise, though slight, were fairly consistent for a given dog but apparently bore no relation to the kind of exercise. In dogs 2 and 5, the pH fell 0.05 during the first 15 minutes of exercise, in the other animals the pH rose approximately the same amount. In all of the dogs, however, a decrease in pH from the 15 minute value was noted in the blood at exhaustion.

Figure 2 shows the acid-base changes with exercise and recovery for dogs 3 and 5 exercised by swimming and also for dog 1 exercised on the treadmill. They are plotted on triaxial graph paper according to the method of Hastings and Steinhaus (11). The chart shows that whether the pH rose or fell within the first 15 minutes of exercise, there was always over-breathing, resulting in a fall in the CO_2 tension, but that with continued exercise to exhaustion very little further change in CO_2 tension occurred. A further fall in bicarbonate concentration can be attributed almost entirely to the entrance of fixed acid into the blood.

The extent to which fixed acids have entered the blood may also be estimated by comparing the bicarbonates of the different samples after correction to constant pH. In making this correction the value 28 has been used for the relation $\frac{\Delta[BHCO_3]_s}{\Delta pH_s}$. Although this is the value found for

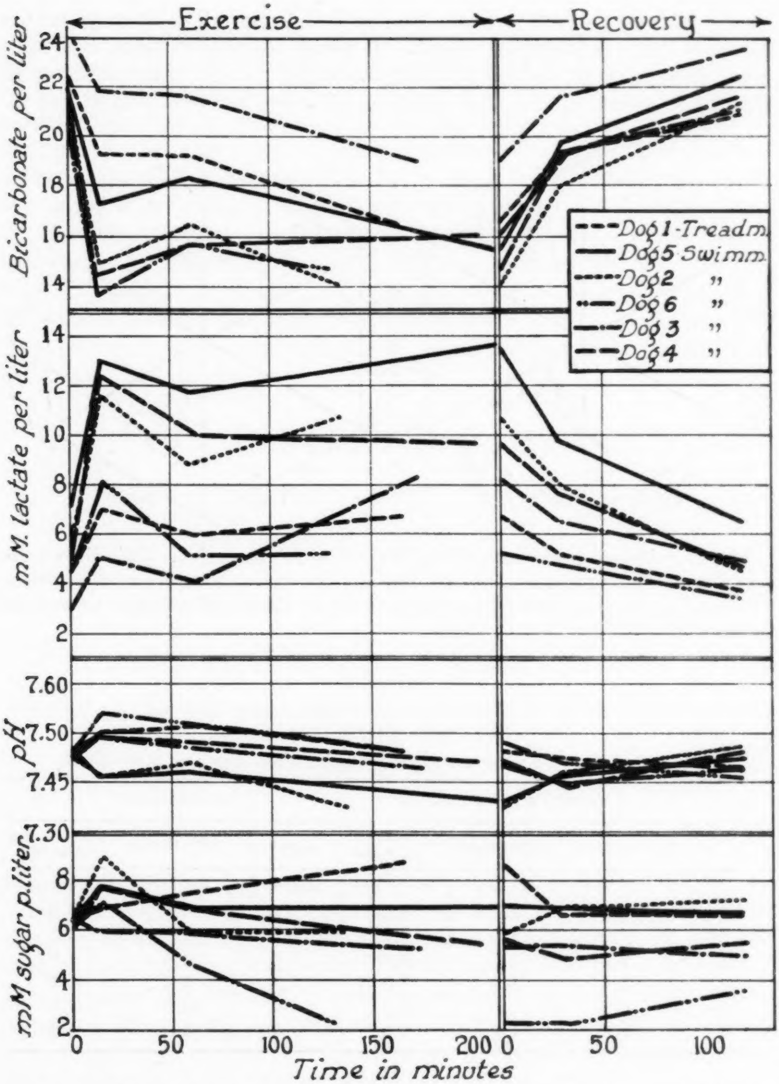

Fig. 1. Effect of exercise to exhaustion upon serum pH and concentrations of bicarbonate, lactate and sugar.

human blood (12), experiments on the buffer value of dog blood did not yield a significantly different value. It is interesting to see how closely the fall in bicarbonate corrected to constant pH (Δ bicarbonate) is related

to the increase in lactate (Δ lactate) in the serum. The average results for each dog are given in table 2. It would seem that there is a close correlation between the fall in bicarbonate and the rise in lactate after 15 minutes of exercise, and that after the effects of over-ventilation are al-

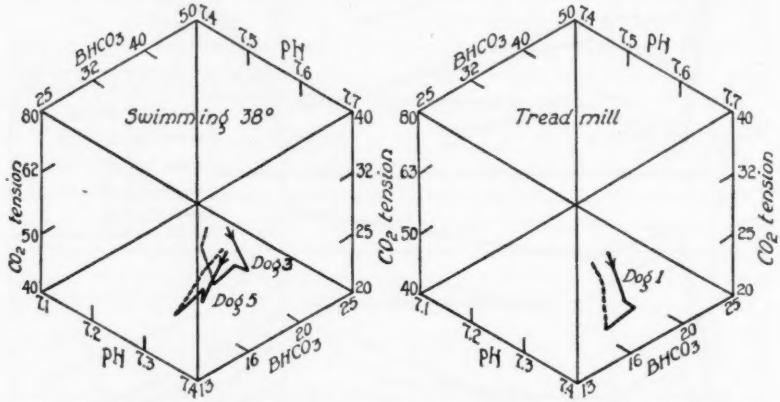

Fig. 2. Acid-base paths accompanying exercise to exhaustion. Solid lines indicate changes during exercise, broken lines changes during recovery after exercise. CO_2 tension is expressed in terms of millimeters of Hg and bicarbonate concentrations as millimols per liter of serum.

TABLE 2

The relation between fall in bicarbonate (corrected to initial pH) and rise in lactate with exercise

Dogs 2, 3, 4, 5 and 6 were exercised by swimming, dog 1 on a treadmill

DOG NUMBER	AFTER 15 MINUTES' EXERCISE			AT EXHAUSTION		
	Δ Lactate	Δ Bicarbonate	Δ Bicarbonate − Δ lactate	Δ Lactate	Δ Bicarbonate	Δ Bicarbonate − Δ lactate
	mM/liter	*mM/liter*	*mM/liter*	*mM/liter*	*mM/liter*	*mM/liter*
1	2.5	2.5	0.0	2.1	6.1	4.0
2	6.0	7.3	1.3	4.1	9.8	5.7
3	2.0	1.5	0.5	5.2	6.2	1.0
4	7.5	5.5	2.0	4.7	5.4	0.7
5	5.5	7.5	2.0	5.9	10.2	4.3
6	3.6	4.9	1.3	0.8	6.0	5.2

lowed for, any additional decrease in bicarbonate concentration may be approximately accounted for by the entrance of lactic acid into the blood. This is also apparently true for dogs 3 and 4 at exhaustion. In the cases of dogs 1, 2, 5 and 6 the fall in bicarbonate at exhaustion is too great to be entirely explained by the increase of lactic acid in the blood.

Since the fall in bicarbonate at exhaustion cannot always be explained by an increase in lactic acid, some additional explanation is required. From the standpoint of acid-base balance, there must be either a compensating decrease in total base, or the increase or appearance of some acid not yet accounted for. In this connection we are making a study of the total acid-base balance of the blood serum before and after exercise. The serum is analyzed for total base and the common acid ions, bicarbonate, chloride, phosphate, lactate and protein (globulin and albumin). Unfortunately the difference we should like to account for is small, i.e., 4 to 6 mM, whereas unavoidable experimental errors in chloride and total base determinations may introduce a difference of 2.5 mM in the balance. The results obtained so far indicate that there is often a decrease in total fixed base with exercise.

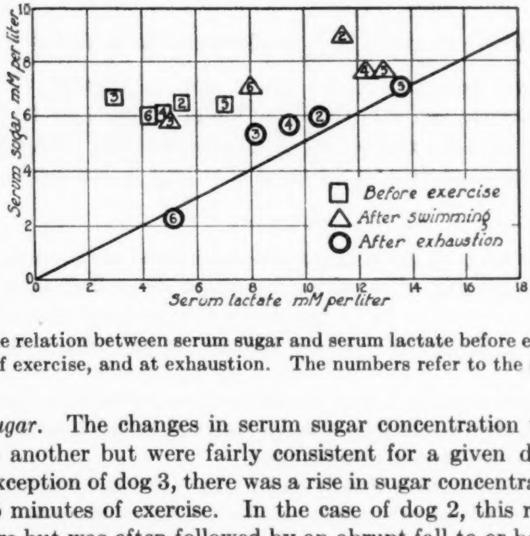

Fig. 3. The relation between serum sugar and serum lactate before exercise, after 15 minutes of exercise, and at exhaustion. The numbers refer to the dogs studied.

Serum sugar. The changes in serum sugar concentration varied from one dog to another but were fairly consistent for a given dog (fig. 1). With the exception of dog 3, there was a rise in sugar concentration during the first 15 minutes of exercise. In the case of dog 2, this rise was unusually large but was often followed by an abrupt fall to or below normal levels as exercise continued. In the case of dog 1, with continued exercise a gradually increasing sugar concentration was found. Dogs 3 and 6, and occasionally dog 2, showed hypoglycemias as exercise continued, that of dog 6 reaching a low average value of 2.3 mM per liter.

Although there appeared to be no correlation between sugar and lactate concentrations at exhaustion in individual experiments, the averages of the experiments on the swimming dogs, 2, 3, 4, 5 and 6, revealed an interesting relationship. This is shown in figure 3 where the serum sugar is plotted against the serum lactates. The average results of the five

dogs at exhaustion (circles) approximate the ratio $\frac{\text{sugar}}{\text{lactate}} = \frac{1}{2}$ which is represented by the solid line.[4] On the other hand, the initial points (squares) and the results found for the 15 minute periods (triangles) lie well above this line. This suggests that, under normal conditions and prior to exhaustion, there is an excess of sugar available for mechanical work; and furthermore that only a fraction of it appears as lactate in the blood; but that at exhaustion the splitting of sugar to lactic acid goes on, with most of the lactic acid entering the blood stream until a limiting condition is set up which is represented by the ratio $\frac{\text{sugar}}{\text{lactate}} = \frac{1}{2}$. If, as occurred in dog 6, the supply of sugar is reduced, the concentration of lactate is correspondingly reduced. If the lactate concentrations found by the gasometric method were decreased to make them correspond to the distillation method, this would have the effect of moving all points to the left. The value of the limiting ratio $\frac{\text{sugar}}{\text{lactate}}$ would therefore be raised but the conclusions drawn from the results would not be affected. Tentatively, then, an additional factor in the definition of fatigue may be postulated, namely, that when the ratio of serum glucose to serum lactate approaches 0.5, a condition conducive to the onset of fatigue is imminent.

SUMMARY

A study of some of the changes which occurred in the blood serum of the normal dog exercised to exhaustion either on an inclined treadmill or by swimming in water at 38°C. has shown that although the response was not invariable, there were certain changes which were quite generally characteristic.

A rise in lactate and a fall in bicarbonate concentration within the first 15 minutes of exercise were invariably observed; subsequently there was a tendency to return toward initial values.

While pH changes were usually slight and varied in direction depending on the dog, the pH at exhaustion usually fell below the previous exercise level. The acid-base changes may be described as over-ventilation, coupled with fixed acid excess. After allowing for the effects of over-ventilation, the fall of bicarbonate after 15 minutes' exercise could be accounted for quantitatively by the increase of lactic acid in the blood; at exhaustion, however, the fall in bicarbonate was often greater than the rise in lactate.

The serum sugar concentrations at the end of 15 minutes of exercise either remained within the normal range or increased; with continued

[4] The results on dog 1 which ran on the treadmill and which was less fatigued by the exercise than by swimming did not show this relation between sugar and lactate.

exercise they tended to fall, sometimes to very low values. Hyperglycemias were always coincident with a high lactate concentration and a shortened time to onset of exhaustion.

A relation was observed between the serum sugar and serum lactate of the dogs who were exercised by swimming at 38°, suggesting that at exhaustion the serum lactate concentration was dependent upon the serum sugar concentration.

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STANDARDS FOR PREDICTING BASAL METABOLISM IN THE IMMEDIATE PRE-ADULT YEARS

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The clinical value of basal metabolism tests in subjects under 21 is not by any means as firmly established as is the case with adults, largely because of the confusion that still exists among so-called normal standards of reference for the pre-adult years. This lack of agreement is understandable in the light of the additional problems involved in both the making of truly basal measurements on the younger subjects, and also in the decisions necessary as to how the determined metabolism should best be referred to one or more of the body measurements to afford the most valid prediction basis from groups to individuals. Thus there have been decided gaps in the older normal data, and methods of building standards for tentatively filling in these gaps have been contradictory and confusing in practical application.

Because of this confusion among prediction standards for the latter half of the second decade, a systematic study of the age-range 17 to 21 was undertaken at the University of Wisconsin some years ago. This relatively narrow but practically important group represents one of the gaps in our knowledge of physiological normals referred to above. Normal subjects of these ages are surprisingly poorly represented in the previously reported studies of basal metabolism when one considers that such subjects are mature enough to give readily the necessary type of coöperation. However, studies dealing with older children have been concerned for the most part with ages up to about 16, while the vast majority of normal adults studied have been over 21.

The ages between 16 and 21 constitute a borderline period in which body growth continues to a small degree in many individuals. Whether the metabolism for this period should be predicted by the same methods that have proven suitable for adults, or whether such subjects must be considered along with growing children, has been tentatively decided in different ways by different investigators.

Thus, for example, Benedict extrapolated up to 21 his curves for girls of ages 12 to 17 (1, 2), whereas others have preferred to predict for both

Metabolism measurements on 62 men, ages 17–21, at the University of Wisconsin[*]

NUMBER	AGE: YEAR AND MONTH	WEIGHT, KGM.	HEIGHT, CM.	CAL./24 HRS.	CAL./SQ.M./HR.	NUMBER	AGE: YEAR AND MONTH	WEIGHT, KGM.	HEIGHT, CM.	CAL./24 HRS.	CAL./SQ.M./HR.	NUMBER	AGE: YEAR AND MONTH	WEIGHT, KGM.	HEIGHT, CM.	CAL./24 HRS.	CAL./SQ.M./HR.
1	16^{7}	57.5	176	1,616	39.6	22	17^{11}	66.6	176	1,790	41.2	43[a]	19^{0}	75.2	181	1,979	42.3
	16^{7}	57.8	176	1,602	39.3		17^{11}	67.5	176	1,688	38.6		19^{0}	74.9	181	2,007	42.9
2	16^{7}	56.1	173	1,582	39.5	23	18^{1}	73.3	183	1,939	41.7	44	19^{1}	80.7	181	1,713	35.5
	16^{7}	56.1	173	1,597	39.8		18^{1}	74.2	183	1,884	40.3		19^{2}	80.0	181	1,771	36.9
3	16^{9}	66.8	181	1,809	40.7	24	18^{2}	49.4	166	1,545	42.1	45	19^{1}	68.4	177	1,612	36.5
	16^{8}	66.6	181	1,848	41.6		18^{3}	50.0	166	1,456	39.4		19^{3}	70.1	177	1,637	36.5
4	16^{8}	54.6	172	1,655	42.6	25	18^{2}	64.5	184	1,834	41.3	46	19^{2}	59.8	172	1,488	36.5
	16^{8}	55.0	172	1,550	39.4		18^{2}	63.4	184	1,750	39.9		19^{2}	60.3	172	1,560	38.0
5[a]	16^{9}	97.7	190	2,380	44.1	26	18^{2}	54.8	173	1,577	39.8	47	19^{3}	79.1	185	1,840	37.8
	16^{10}	98.5	190	2,490	45.9		18^{3}	54.9	173	1,613	40.7		19^{3}	79.7	185	1,716	35.2
6	16^{10}	49.8	169	1,373	36.9	27	18^{3}	53.8	168	1,324	34.5	48	19^{4}	64.0	175	1,741	41.0
	16^{10}	49.5	169	1,387	37.3		18^{3}	55.3	168	1,438	37.0		19^{4}	63.1	175	1,698	40.2
7	16^{11}	74.9	183	1,778	37.8	28	18^{3}	71.5	176	1,880	41.9	49	19^{5}	74.5	172	1,759	39.2
	17^{0}	75.9	183	1,840	38.9		18^{3}	72.4	176	1,817	40.3		19^{5}	74.4	172	1,601	35.7
8	17^{0}	66.3	176	1,675	38.8	29	18^{4}	58.7	173	1,643	40.3	50	19^{8}	71.9	180	1,884	41.3
	17^{0}	66.3	176	1,664	38.5		18^{4}	59.1	173	1,658	40.6		19^{8}	72.3	180	1,931	42.4
9	17^{0}	58.5	180	1,751	41.9	30	18^{4}	71.5	180	1,733	38.0	51	19^{8}	64.4	170	1,750	41.9
	17^{0}	59.0	180	1,566	37.5		18^{4}	72.4	180	1,856	40.5		19^{9}	62.7	170	1,680	40.7
10	17^{1}	60.7	174	1,577	38.2	31	18^{5}	71.5	183	1,793	38.9	52[a]	19^{11}	83.5	191	2,047	40.0
	17^{1}	60.6	174	1,545	37.4		18^{5}	72.1	183	1,581	34.1		19^{11}	84.3	191	1,973	38.6
11	17^{2}	59.4	181	1,786	42.3	32[a]	18^{6+}	62.2	172	1,466	35.3	53	19^{11}	51.8	177	1,559	39.6
	17^{2}	61.0	181	1,684	39.4		18^{6+}	63.4	172	1,581	37.9		19^{11}	52.0	177	1,432	36.4
12[a]	17^{2}	72.7	185	1,976	42.2	33	18^{7}	85.3	188	1,929	37.9	54	19^{11}	77.8	189	1,879	38.4
	17^{2}	72.4	185	1,924	41.1		18^{8}	84.6	188	1,771	35.0		20^{0}	77.5	189	1,817	37.1
13[a]	17^{3}	78.5	187	1,842	37.6	34	18^{7}	69.1	177	1,887	42.5	55	20^{2}	66.5	186	1,581	34.9
	17^{3}	77.4	187	1,845	38.1		18^{10}	68.8	177	1,773	39.9		20^{2}	66.4	186	1,650	36.4
14	17^{5}	69.5	183	1,895	41.6	35	18^{8}	61.1	171	1,645	40.1	56	20^{2}	72.9	191	1,819	37.9
	17^{5}	69.8	183	1,744	38.3		18^{8}	60.6	171	1,684	41.3		20^{5}	74.9	191	1,801	37.2
15	17^{5}	61.0	180	1,590	37.4	36	18^{8}	67.3	177	1,729	39.4	57	20^{3}	84.4	198	1,896	36.6
	17^{5}	62.1	180	1,553	36.2		18^{8}	68.4	177	1,733	39.2		20^{3}	84.1	198	1,830	35.0
16	17^{7}	65.6	179	1,677	38.4	37	18^{9}	72.3	178	1,584	34.8	58	20^{4}	67.7	178	1,634	37.2
	17^{7}	66.3	179	1,604	36.5		18^{9}	72.7	178	1,797	39.4		20^{4}	67.1	178	1,641	37.4
17	17^{7}	67.0	177	1,849	42.3	38	18^{10}	59.2	167	1,646	41.3	59	20^{4}	70.4	178	1,677	37.2
	17^{11}	69.3	177	1,800	40.5		18^{10}	60.2	167	1,574	39.0		20^{4}	70.5	178	1,535	34.0
18	17^{8}	56.3	175	1,549	38.4	39[a]	18^{10}	79.4	183	2,064	42.6	60[a]	20^{4}	56.2	161	1,409	37.2
	17^{8}	57.0	175	1,525	37.6		18^{10}	77.5	183	2,164	45.1		20^{4}	56.1	161	1,298	34.2
19	17^{9}	72.5	177	1,806	39.8	40	18^{10}	99.0	203^{+}	2,300	40.1	61[a]	20^{4}	93.7	189	2,163	40.8
	17^{9}	71.8	177	1,838	40.7		18^{10}	99.4	203^{+}	2,251	39.2		20^{4}	94.5	189	2,067	38.8
20	17^{9}	75.0	180	1,869	40.1	41[a]	18^{11}	81.5	192	2,198	43.4	62	20^{5}	64.6	169	1,590	38.1
	17^{10}	74.3	180	1,879	40.6		19^{0}	80.6	192	2,009	39.9		20^{5}	65.3	169	1,640	39.1
21	17^{10}	74.5	185	1,929	40.8	42	19^{0}	64.2	177	1,587	36.9						
	17^{10}	75.8	186	1,946	40.8		19^{0}	64.7	177	1,616	37.4						

[*] Data for Wisconsin girls of the same ages are reported in Ref. 3, a.

older boys and girls in the same way as for adults. The serious conflict resulting from these diverse methods in the case of girls of 17 to 21 has been dealt with in earlier papers from this laboratory (3a, b), wherein a statistically significant number of normal data were offered to justify some rather extensive analyses and comparisons. Though the conflict in prediction standards for older boys has never been so serious as was the case with girls, a relative dearth of actual data for these particular years was still in evidence. To help to fill this in for future standard-builders, and to afford a basis for some strictly parallel comparisons between the metabolism findings for the two sexes, the Wisconsin study was therefore extended to the present series.

Experimental. *The subjects.* Only subjects in good health were accepted for the study, as indicated by a grade of A in their physical examinations through the Student Health Department, and further by their condition at the time of the tests.

All but one of the 62 boys studied were from goiter-belt regions (4) and all but 5 were Wisconsin residents, thus presenting a more homogeneous group than the 97 girls previously reported. The metabolism of the latter, however, had appeared entirely comparable with that of other healthy subjects of the same ages being reported from various non-goiter-belt sources (3).

The tests were made by the same operator and with the same technique as was the case with the girls of the parallel study,—i.e., the Sanborn-Benedict clinical apparatus was employed, with the usual precautions as to the securing of "basal" conditions. All tests were run in duplicate and each subject included in the final series had two technically acceptable tests on different days. Here again, as in the study of girls, there was no attempt to control the dietary or other régimes under which the subjects lived, since as representative a sample as possible of the University at large was desired for general reference purposes.

The fundamental data on the 62 boys comprising the present series are given on page 631, and the average measurements with their standard deviations in table 1. The total series of boys is smaller than the corresponding one for girls, both because it was more difficult to obtain the male volunteers, and because fewer data were necessary to establish agreement with existing standards. The numbers of the two who had more than one test apiece, however, were almost identical,—i.e., 64 girls and 62 boys.

Comparative studies on the data for boys and girls. Stability: Measurements of oxygen-consumption obtained on different days on the same subjects agreed within 5 per cent for 63 per cent of the boys and 64 per cent of the girls. However, the tendency toward lower values in tests after the first ones—accepted, other things being equal, as pointing to decreasing nervousness—is definitely greater with the boys than the girls. The

corresponding ratios between decreases and increases in the rate of oxygen used in tests after the first were 40:30 for girls, as against 39:23 for boys. This is the opposite of the experience usually reported with adults (5, et al.). The reversal may reflect the fact that the observer here has been a woman, whereas the operators in most of the older studies were men, with the conceivable difference this might make in the ideal state of relaxation of the subjects, depending on their sex. It certainly suggests, at the least, a psychic factor of a type that is readily overlooked in attempting to make

TABLE 1

Average measurements and basal metabolism of 62 boys, ages 17 to 21. First and later tests are given separately

AGE	NUMBER	WEIGHT, KGM.		HEIGHT, CM.		CALORIES PER 24 HOURS		CALORIES PER SQ. M./HR.		DIFFERENCE BETWEEN DUPLICATE O_2 MEASURED WITHIN TESTS, AS PER CENT OF LOW RUN*
		Average	σ	Average	σ	Average	σ	Average	σ	
17	15	65.5	11.50	179.2	5.80	1,753	222.4	40.1	2.12	2.3
		65.9	11.67	179.2	5.86	1,722	249.8	39.2	2.28	1.9
18	16	65.4	8.05	177.2	5.25	1,734	163.7	40.0	1.95	2.9
		66.0	8.01	177.3	5.38	1,708	155.4	39.3	1.90	2.5
19	17	72.2	10.52	179.7	8.55	1,789	234.3	39.1	2.77	2.2
		72.3	10.24	179.8	8.48	1,785	199.0	39.0	2.50	2.1
20	14	71.5	10.74	180.5	10.23	1,761	197.1	38.6	1.96	3.1
		71.6	10.98	180.6	10.22	1,707	204.1	37.3	2.30	2.7
17–20	62	68.7	10.75	179.1	7.78	1,759	207.7	39.4	1.89	2.6
		69.0	10.71	179.2	7.78	1,732	206.6	38.8	2.42	2.3

* The lower of duplicate runs within any given test is accepted for that test, and the difference between the duplicate runs expressed as percentage of this figure. This gives a higher result than if the difference were expressed as percentage of the average, as is often the case.

physiological comparisons between "basal" data obtained under different conditions.

Because of the definite suggestion of improved relaxation in the later tests of the boys *as a group*, we have from now on centered our attention upon tests after the first ones, as being the more representative in making group comparisons.

Pulse rates here as usual ran only roughly parallel to the metabolic measurements. The rates, summarized in table 2, show a rather surpris-

ing incidence of bradycardia among these healthy young men, with rates lower than might be expected also among the girls. In the men's series, pulses under 55 per minute were more than twice as common among the small group of known athletes mostly in training for competitive sports at the time of the tests, as among the remainder of the group. The metabolic level of these boys, particularly the heaviest ones, was more often above than below the group average, suggesting a relationship with muscular development and tonus. These subjects are distinguished by the superscript "a" in the data table. Occasionally, as with subject 27 in the first test and subject 47 in the second test, relatively low pulse rates with low metabolism accompanied the depression of excessive fatigue.

TABLE 2

Basal pulse rates

	AGE	NUMBER OF SUBJECTS	BASAL PULSE RATES			
			Range in first tests	Range in later tests	Average in first tests	Average in later tests
Boys	17	15	40–84	42–76	64	61
	18	16	48–76	48–78	63	64
	19	17	44–76	43–74	61	58
	20	14	41–84	43–86	64	63
	17–20	62	40–84	42–86	63	61
Girls	17	15	(48–86)*		70	68
	18	24	(53–86)		69	69
	19	11	(43–89)		71	63
	20	14	(59–88)		71	69
	17–20	64	(43–89)		70	68

* Taken from the tables for all 97 subjects of the original girls' series. The rest of the figures,—i.e., the averages, have been recalculated for the 64 who had more than one test.

The measured metabolism and the fit of the different "normal" standards for ages 17 to 21. The average calories found in tests after the first in the Wisconsin series of boys and girls are given in table 3 as total calories per 24 hours, and as calories per unit of weight, of height and of surface area. The table also presents comparable measures of the prediction accuracy of various classical standards (6, 7, 8, 1) applied to both boys and girls.

Three points of comparison are included: 1, the mean "rate" of the group according to the given prediction;[1] 2, the extreme range of the calcu-

[1] The calculated individual "rates" express, as is conventional, the percentage deviations of the measured values from a given prediction, using the latter as 100 per cent.

lated rates; and 3, the statistical measure of scatter expressed in the standard deviation of the individual calculated rates. These indicate respectively the relative absolute levels at which the predictions are set; the magnitude of spread on either side of the zero-points of these standards; and finally the measure of prediction accuracy which is more fundamental

TABLE 3

*Average calories of heat-production for boys and girls, 17 to 21, and success of different standards in predicting metabolic rates**

	GIRLS	BOYS	SEX DIFFERENCE (PER CENT OF MALE VALUE)
Number of subjects	64	62	
Average determined calories:			
Total per 24 hours	1,291	1,732	25
Per kgm. per 24 hours	23.16	25.12	8
Per cm. per 24 hours	7.91	9.67	18
Per square M. per hour	33.70	38.75	13

	GIRLS			BOYS		
	Algebraic mean of calculated "rates"	Standard deviation, corrected predictions†	Extreme range of calculated "rates"	Algebraic mean of calculated "rates"	Standard deviation, corrected predictions†	Extreme range of calculated "rates"
Prediction standards:						
Harris-Benedict adult (7) extrapolated for age	−8.1	6.88	−22..+12	−3.1	5.87	−15..+13
Aub-DuBois (6)	−12.4	7.44	−28.. +7	−6.7	6.25	−19..+10
Dreyer (8)	−9.0	7.11	−22..+12	−1.1	6.39	−17..+18
Wisc. pred., girls 17–21 (3)	−1.0	6.90	−16..+21			
Benedict, girls 12–21 (1)	+7.0	9.07	−11..+34			

* Based on tests after the first, only, in all cases.

† For fair comparison in deriving these figures *only* (the rest are with the predictions as they stand) each prediction has been corrected by the constant percentage amount necessary to center it for the Wisconsin series. See text.

than either of these, afforded by the standard deviation of the individual differences between measured and predicted values.

For strict statistical fairness, this last comparison has been made after the differences in average absolute level of the predictions have been allowed for by correcting each by the constant amount necessary to "center"

them all to the same mean. The need for this is the fact that any given difference in calories would constitute a different percentage of a standard which happens to be set on the average too high, than of one which is set too low for the average of a given group. This is effectively the same method used by Jenkins (9) in his recent comparisons among numerous earlier recorded data (including very few for these particular ages), excepting that, to simplify calculations, he reversed the usual procedure of comparing the individual measurements to a given standard, using the latter as 100 per cent, and referred instead the different standards to the chosen bodies of data.

Table 3 shows the superior success of the Harris-Benedict standard in predicting for both boys and girls of these ages, though the original standard was designated for ages down to 21, only. Benedict (2) has recognized that his adult standard can safely be extrapolated for boys under 21, but has not, apparently, given his official sanction to this extension, preferring to wait for further data on younger normals. The standard when extrapolated in a straight line has been superior here not only as it stands, but fundamentally the most successful in grouping the individual data about their mean, as shown by the standard deviations, corrected as before described. The differences between the three classical predictions for boys here is very small, but for girls it is much more significant. The fact that available predictions for males are better than any of those offered for females is emphasized by these comparisons, justifying the formulation of the Wisconsin prediction, which was offered for temporary practical use and comparisons only. Of course eventual permanent amendment of the classical standards must be based on the study of a great many groups of normals of all ages and from many localities. A poorer, rather than a better fit for the Wisconsin data is afforded by either Boothby and Sandiford's modifications of the Aub-DuBois predictions (10) or by Kestner and Knipping's modified extensions of the Harris-Benedict tables (11).

The sex difference: By table 3 it is seen that the sex difference in heat-production for the two Wisconsin groups is 25 per cent in the average total calories, and 8 per cent, 18 per cent and 13 per cent (using always the male values as standard) for calories per unit respectively of body weight, height, or surface area. DuBois tentatively (12, p. 169) built his prediction for females by subtracting 7 per cent all along the line from the average calories per square meter of surface area derived from his smoothed curves for males. The difference actually found here is 13 per cent, and as a result there is a 6 per cent greater discrepancy in the fit of DuBois' standard for our girls than for our boys.

In tabulating values for the Dreyer prediction, Stoner (8b) listed only the figures for males and specified the female prediction to be derived by subtracting 10 per cent from the values for males of the corresponding weights

and ages. Dreyer's formula includes the square root of weight in a logarithmic relationship (8a) so that the suitability of this 10 per cent sex difference assumed cannot be seen directly, but must be tested by substituting average values for weight and age in the original formula. When this is done for these groups, it appears that the difference should be more like 13 per cent than the 10 per cent specified by Stoner.

There is of course no justification in the study of any one age-range for assuming that this sex difference would run parallel throughout a large

TABLE 4

*Coefficients of correlation of measurements and measures of success in predicting metabolism by equations using weight and height, or weight alone. Wisconsin boys, 17 to 21**

Number of subjects	62
Coefficients of correlation:	
Calories and weight	0.849 ±0.024
Calories and height	0.734 ±0.039
Weight and height	0.816 ±0.028

	COEFFICIENT OF ALIENATION†	STANDARD ERROR OF ESTIMATED METABOLIC RATES	
		In calories	In per cent
(a) When weight and height are both included in the regression equation: (Cal. = 14.44 Wt. + 3.27 Ht. + 149.75)	0.524	108	6.2
(b) When only weight is considered in the regression equation: (Cal. = 16.39 Wt. + 601.89)	0.528	109	6.3

* Tests after the first ones, only, are included in each case.

† Coefficient of alienation represents the percentage of error, compared to that of a prediction consisting merely of the group average, which remains when additional variables—here weight, or weight and height—are included in the prediction equation. It is desirable, therefore, to keep this coefficient (as is the case for the standard error of estimate) as small as possible.

range of observed heights, weights and ages. There would seem to be no substitute for the separate study of all the different age-ranges of both sexes in the search for accuracy in prediction standards.

The importance of including height in formulas for predicting basal metabolism is not at all as unquestioned as the need for including weight. Thus Dreyer, as quoted by Stoner (8b: first ref.) considers height an "erroneous biometric measurement" and achieves an admirable degree of success with a formula which leaves it entirely out of consideration. On the other hand, in the age-range up to 12, the heat-production apparently

shows more nearly a direct proportionality with height than it does with weight (13). DuBois has stressed the desirability of further study of this point (14).

It was hoped that this series of boys would help to throw some light on this question since it represents an unusually wide range of heights. The actual range is from 160.5 cm. (or 63 inches) in subject 60, to 203.5 cm. (or over 80 inches) in subject 40. In Benedict's second male series (5) which purposely included a wide range of body sizes, he noted that two men had "the unusual height of 188 centimeters."

Prediction equations were formulated therefore for our boys by the method of multiple linear regression, to include *a*, both weight and height; *b*, weight alone; and *c*, height alone. These equations are given and the success of the first two of them is compared statistically in table 4. Both the "coefficients of alienation" (explained with the table) and the standard errors of estimating the rates by the different equations show that practically nothing has been gained in prediction accuracy when height is considered in addition to weight. (Height alone is not as successful as weight alone, and has therefore been left out.) The reason for this lack of improvement in a series such as this is doubtless the fact—seen in the correlation coefficients given in the same table—that these young men are predominantly too *typical* in build. That is, very little information about their heat-production is gained by knowing the heights, that would not be similarly available in the knowledge of their weights, since weight and height are so closely related to each other.

Even when one considers those *individuals* of the group who represent the two extremes of weight-for-height relationship,[2] no clear-cut advantage of any one of the three types of prediction equation is discovered. On the whole it is merely suggested that weight alone has predicted better than height alone, or even than weight plus height, for most of the underweight subjects, whereas height appears desirable in predicting for the overweight, as we might expect.

The two groups of atypical weight-for-height do not include any of the four tallest men in the series, whose weights all happen to fall within 7 per cent (three of them within 3 per cent) of those predicted by the group equation for their respective heights. These are subjects 40, 57, 41 and 52, whose heights range from 203.5 to 191.5 cm. The calculated metabolic rates of the four all appear "normal,"—i.e., they fall within the range of ± 0 and -12 when calculated according to the Harris-Benedict prediction (average for the whole group: -3); and between $+7$ and -5 according to Dreyer (group average: -1). There is nothing to be learned

[2] Subjects 5, 44, 49, 60, 61 and 62 are from 11 to 22 per cent overweight; and subjects 6, 9, 11, 15, 18, 25, 53 and 55 are from 13 to 22 per cent underweight, according to the weight-for-height relationship that applies to the group as a whole.

by comparing the three different Wisconsin predictions in table 4 for these four subjects. Different methods than the study of such essentially normal groups are evidently necessary to throw further light upon the fundamental heat-height relationship.

DISCUSSION. Returning to the Wisconsin young men and women as a whole, then, the success of the Harris-Benedict and of the Wisconsin predictions indicates an essentially linear relationship between the heat-production and body size for the ages 16½ to 20½. On the other hand, the relative lack of success in grouping the data by Benedict's special standard for girls (3, and table 3, above) shows that the metabolism at this stage of development quite definitely should not be looked upon as a simple function of the body weight. The difficulties of assuming such a simple relationship are exaggerated when we attempt to predict for individuals of definitely abnormal body build, as is illustrated by an example that happens to be at hand. This is B.C., a 17 year old girl sent in for study from the University High School because of her small size. She is 141 cm. tall and weighs 32.3 kgm., and her basal calories are reported as 1147 per 24 hours in the lower of well-duplicated tests. This gives her a metabolic "rate" of -5 according to Dreyer, -4 according to DuBois, ± 0 by Harris-Benedict, but of $+63$ by Benedict's special prediction for girls.

A further study has been in progress here for a number of years and its continuation is planned, to trace the progressive *changes* in heat-production that accompany changing body proportions in a group of growing children of diversified body builds. Perhaps studies such as this of long-time individual curves may open the way to an improved understanding of the factors that need to be considered in arriving at the best possible basis of predicting the heat-production from the various body measurements.

SUMMARY

Basal metabolism tests in duplicate are reported on 62 boys of ages 17 through 20 from the University of Wisconsin and the University High School. This series supplements a previous Wisconsin study of girls of the same ages and permits some generalizations and comparisons between the two sexes.

Statistical comparisons of available prediction standards show that the Harris-Benedict adult prediction extrapolated in a straight line for these ages is the most successful for both sexes.

The present series of men represents an unusually wide range of heights. But probably because the group is too homogeneous in weight-for-height relationship, there is no significant gain in prediction accuracy when height and weight both are included in the prediction equation, over that

attained by using weight alone. A better understanding of fundamental relationships must perhaps await different types of study.

The present study indicates that within the latter half of the second decade the relationship of metabolism to body size is essentially linear in type, though there is distinctly not a direct simple proportionality between heat-production and body weight. An example of abnormal body build illustrates the possibility of grave misconceptions that can arise from the use of a prediction standard that makes the latter assumption.

Acknowledgments. Grateful acknowledgment is hereby made to the students who gave the time and trouble to coöperate as subjects, and to members of the Departments of Student Health, of Medical Physiology, and of the University High School for facilitating contacts with suitable material. Gratitude is also due to Prof. Mark Ingraham of the Department of Mathematics for aid in the statistical aspects of the analysis of data, and to Miss Beatrice Berberich, who performed the most exacting of the calculations. Dr. E. L. Sevringhaus of the Department of Medicine has been relied upon continuously as a consultant during the study.

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THE NATURE OF THE ACTION POTENTIALS IN THE FROG'S GASTROCNEMIUS MUSCLE[1]

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In a recent communication (1) we have presented an analysis of the R wave potentials in the heart of the tortoise and frog. The method employed was to record the potentials from the heart at different points in a conducting field of a form in which the theoretical distribution of potentials due to charges near the center is known, and to compare this theoretical with the actual distribution determined with the heart in such a field. It was found that the electrical change within the heart, responsible for the initial potentials in the ventricular potential complex, is that of two asynchronous dipoles or pairs of associated positive and negative charges developing and declining at different time intervals. The axes of the two dipoles are nearly at right angles, but oriented in general with the negative charge toward the base, the positive charge toward the apex of the ventricle.

The present report concerns the result from similar experiments carried out with the gastrocnemius muscle of the frog, stimulated through its motor nerve. The principal object of this work was to obtain a preparation in which the distribution of action potentials, particularly as regards symmetry, is presumably simpler than in the case of the heart. The preparation one would choose in this connection would be that of a long parallel-fibered muscle, stimulated locally at one end, and probably curarized to prevent the possibility of uneven distribution of the impulse through motor nerve terminals. Technical difficulties, connected with stimulus escape have, however, prevented us up to the present time from obtaining satisfactory results with a preparation of this type.[2]

[1] Supported in part by grants from the Wisconsin Alumni Research Foundation and the National Research Council.

[2] The asymmetry of the gastrocnemius muscle is related to the presence of two tendons at the proximal end and to a larger mass of muscle posterior to the central tendon than anterior to this structure (2). The two tendons form an acute angle with each other and unite to form a single central tendon somewhat above the mid-region of the muscle. Muscle bundles pass obliquely down and outward, forming acute angles with the tendons. Near the lower end of the central tendon, the bundles pass straight down to the Achilles tendon. Regarded from either the dorsal or ventral aspects, the muscle is nearly symmetrical. Each muscle fiber receives only one branch from a motor nerve fiber, although this breaks up into a number of branches which run nearly the whole length of the fiber (3).

METHODS AND EXPERIMENTAL PROCEDURE. Reference is to be made to the previous publication (1) concerning ventricular potentials, where these are described in detail. The only differences in the present experiments were the use of artificial stimulation and the employment of higher speed in photographic recording in order to analyze the more rapid action potential curves of skeletal muscle. Avoidance of stimulus escape to the conducting field in which the muscle was placed was secured by having a long stretch of motor nerve and stimulating with a low voltage (0.6 volt) condenser discharge at a point on the nerve as far as possible from the muscle. The muscle was placed at the center of a circular disc of Ringer's solution and the nerve was held by the stimulating electrodes in the air above the conducting field. Under these circumstances no detectable stimulus escape occurred. The absence of any effect of the stimulating current on the field surrounding the muscle was proven in several ways. No potentials could be detected in the field when either the nerve or muscle was killed or when a wet thread was substituted for the nerve, so long as the stimulating current was not excessive. There can be no doubt that with the method employed the true action potential distribution in the surrounding field was obtained.

The data from the potential curves were studied in the same manner as in the previous work on the heart. This included plotting of the actual potential values along different axes in relation to the long axis of the muscle and at different distances from it, the determination of the type of field represented by equipotential lines and the analysis of the wave form of the potentials along the various axes. As in the work on the heart, an electromyogram was obtained by direct leads from the muscle, recorded simultaneously with the potential curves from different points in the field. The former served as a record of the potential at its source and as a time reference for the potential curves from the field.

EXPERIMENTAL RESULTS. The potential distribution around the contracting gastrocnemius muscle appears simpler in a number of ways than in the case of the heart. Synchronous potentials, plotted at any instant during the course of the potential curve, give a distribution, within the errors of experimental measurement, according with that of a dipole or series of dipoles oriented along the anatomical long axis of the muscle. The potential along the cross axis is zero, and maximum values of potential are obtained along the axis corresponding to the long axis of the muscle. The potential curves along all axes, except the zero axis, are diphasic and similar in form (fig. 1). The initial phase is about one-sixth the magnitude and one-third the duration of the final phase. In the two quadrants surrounding the upper or femoral half of the muscle the initial phase represents a fall, the final phase a rise of potential. In the two quadrants surrounding the lower half of the muscle, the initial potential is positive, the final

negative. Along opposite axes, the potential curves are mirror pictures of each other. There are no axes, as in the case of the heart, which show monophasic curves. There is also no progression in time of wave crests along the different axes, so far as can be determined from our records.

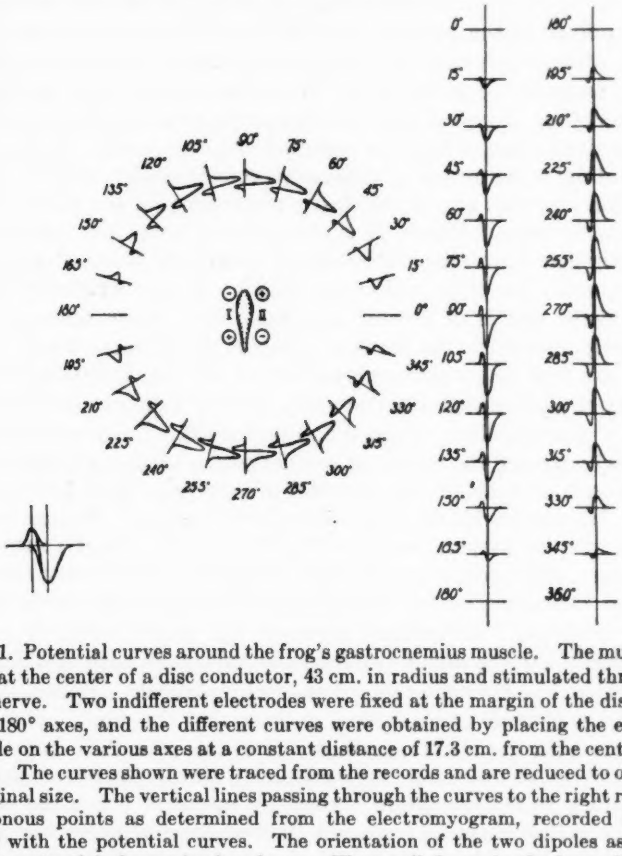

Fig. 1. Potential curves around the frog's gastrocnemius muscle. The muscle was placed at the center of a disc conductor, 43 cm. in radius and stimulated through its motor nerve. Two indifferent electrodes were fixed at the margin of the disc on the 0° and 180° axes, and the different curves were obtained by placing the exploring electrode on the various axes at a constant distance of 17.3 cm. from the center of the muscle. The curves shown were traced from the records and are reduced to one-third the original size. The vertical lines passing through the curves to the right represent synchronous points as determined from the electromyogram, recorded simultaneously with the potential curves. The orientation of the two dipoles associated with the potential change is also shown. The small figure in the lower left hand corner indicates how the typical potential curve may be formed by the summation of two curves of similar form, both being characterized by rapid development and slower decline.

The total duration of the diphasic curve is approximately 0.1 second, of the same order as the duration of the contraction and relaxation periods of the muscle. The changes in amplitude of the initial and final phases of the diphasic curves are proportional throughout.

Discussion. The action potential of the intact gastrocnemius muscle, stimulated through its motor nerve, is composed of two opposite, asynchronous and unequal electrical phases. In the initial phase the proximal end is negative with respect to the distal end, and this is succeeded by a longer period in which the orientation of charges is reversed and in which the magnitude of the potential developed is several times greater. This accords with the effect of two dipoles or groups of positive and negative charges as indicated in figure 1. These two dipoles have the following characteristics. Dipole I is oriented along the long axis of the muscle with the negative charge toward the proximal end, the positive charge toward the distal end of the muscle. It develops coincidentally with the onset of the action potential and is principally responsible for the initial phase of the diphasic curve. Dipole II is also oriented along the long axis, but oppositely placed with its positive charge toward the proximal, its negative charge toward the distal end of the muscle. It develops later than the initial dipole and is of greater magnitude and longer duration. It is principally responsible for the final phase of the diphasic curves.

The view that action potentials in skeletal and cardiac muscle are fundamentally of dipole nature has previously received support from the work of Craib (4) and of Wilson, Macleod and Barker (5). Craib recorded action currents by placing the muscle in contact with a conducting field and connecting one lead to the muscle, the other to some point in the field surrounding it. He thus varied the usual technique of having both leads in direct contact with the preparation suspended in air and without a surrounding conductor. Craib found considerable variations in the form of the curves obtained, depending on the type of the conducting field and the position of the electrodes. In general, however, the curves could be resolved theoretically into the sum of two fundamental curves, each of which might be considered as the expression of an electrical dipole. Wilson, Macleod and Barker showed by theoretical analysis that the dipole hypothesis is consistent with the membrane theory of action potentials, and that the action current, recorded from the mammalian auricle, can be so interpreted.

According to the membrane theory, by means of which the origin of action potentials is usually explained, the two stages of membrane polarization and repolarization must be associated with oppositely directed electrical changes. This does not necessarily imply, however, that the positive and negative electricity produced must be equal, since the order, sequence and speed of the two processes may be quite different. As applied to heart muscle, the QRS complex is generally regarded as the electrical change associated with depolarization, the T wave as the electrical change associated with repolarization (4) (5) (6). Wilson, Macleod, Barker and Johnston (7) have recently determined and compared the amount of elec-

tricity produced in these two phases of the electrocardiogram by measuring the areas enclosed by the QRS and T groups of waves respectively as recorded in the three standard leads. By an analogous procedure to that of the determination by use of the Einthoven triangle of the direction and magnitude of the manifest potential, they determined the mean electrical axis and manifest value of electricity during the QRS and T phases. The addition of the two vectors representing these values gives the mean electrical axis and manifest value of QRST. The magnitude of this last vector is determined by the differences in area under the QRS and T complexes, and it is assumed to represent variations in the excitatory process in different parts of the ventricular muscle.

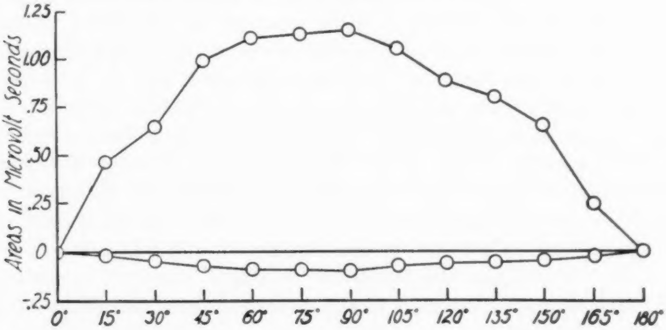

Fig. 2. Areas under the two parts of the diphasic curve recorded along various axes and at a constant distance of 10 cm. from the center of the gastrocnemius muscle placed as described under figure 1. The values are those obtained from potential curves on the axes around the upper half of the muscle. The original curves were enlarged six diameters by optical projection, the areas enclosed determined by a planimeter, and plotted in terms of microvolt-seconds.

An estimate of the quantity of electricity produced during the two phases of the diphasic potential of the gastrocnemius muscle may be made in a similar manner. The areas from a set of potential curves[3] along the various axes around the upper half of the gastrocnemius muscle and at a constant distance from its center are plotted in figure 2. Around the lower half of the muscle the curves would be the same except reversed. From this it appears that approximately ten times as much electricity is produced

[3] The quantity of electricity is defined as the integral of the current and time differential. This corresponds to the area enclosed by the current-time curve. The experimental records are potential-time curves, but since the resistance of the field is constant, these curves are proportional to the current flow, and their integrals will differ from the time quantity of electricity only by a constant factor.

during the final phase as during the initial phase of the diphasic potential. The difference may however not be so great, since the two phases are probably in part fused; i.e., the second dipole probably begins before the complete decline of the first dipole. By the addition of two separate and opposite curves which will approximate the actual curve, it may be shown that the difference must be at least a factor of six.

The plotted values may be regarded as representing the magnitude along the different axes of the components of the two vectors representing the manifest value of electricity in the sense used by Wilson and his co-workers. These fundamental vectors are in this case both along the long axis of the muscle but oppositely directed. The vector sum of the two would give the direction and magnitude of the excess or unbalanced electricity.

While this marked difference in the quantity of electricity developed during the two phases does not exclude the possibility that it is all derived from membrane depolarization and repolarization, and to be explained perhaps by variations in the excitatory process concerned with differences in order, sequence and spread of the two processes, a much more likely explanation appears to us to be the presence of sources of electricity during the second phase other than that derived from membrane changes. The duration of the total diphasic curve is approximately 0.1 second, of the same order as that of the sum of the contraction and relaxation phases of the muscle. The phase corresponding to the T phase of heart muscle would thus comprise all or part of the second phase of the diphasic curve of the skeletal muscle.[4]

The probability that there are other sources of electricity in muscle than that resulting from membrane depolarization and repolarization appears to have had little consideration. The "contraction potential" described by Buchtal (10) from single skeletal muscle fibers, which persists for as long as two or three minutes after the contraction, would appear to be an injury potential produced by the stimulation of the fiber. Many of the processes known to be associated with muscle activity, such as the interchange of water through membranes, change in concentration of electro-

[4] A separate T wave in skeletal muscle, following a more or less definite isoelectric period, such as is consistently observed in cardiac muscle, has been found occasionally in curves made by direct leads from the muscle. The complicating factors under such conditions, relative to the position and distance apart of the electrodes, the number of fibers in activity and the presence of shunting circuits through inactive fibers (8) (9) are such as to make extremely difficult or impossible valid interpretation of the curves obtained, and they may assume various forms under different conditions. Under the conditions of our experiments, these variable factors are avoided and there is no evidence of a separate T deflection. Since the diphasic potential curve coincides approximately with that of the curve of mechanical activity of the muscle, a separate T wave would necessarily fall well after the relaxation phase.

lytes, alteration of acid base equilibrium and oxidation-reduction phenomena are processes invariably associated with the development of electricity. It appears to us inevitable that they should play a rôle in the potential developed by active muscle, and that this potential has a diverse origin, in which membrane potentials play only a part.

The curves of potential indicate two successive processes, similar in form but of opposite polarity and of different duration and magnitude. While superficially the two monophasic potential changes appear to differ in so far as their relative rate of growth and decline is concerned, it is evident that if the second potential develops before the complete decline of the first, the summated effect would give the type of curve obtained (fig. 1), both phases being characterized by a rapid growth and slower decline.[5]

CONCLUSION

The potential change in the frog's gastrocnemius muscle, stimulated through its motor nerve, consists of an initial phase in which the proximal end of the muscle shows a fall, the distal end a rise of potential, and a final phase in which the potential relations are reversed. The final phase is approximately three times as long and six times the magnitude of the initial phase. This accords with the potential associated with two dipoles, or groups of positive and negative charges, oriented along the anatomical long axis of the muscle, of opposite polarity, asynchronous in development and of different magnitude. At least six times as much electricity is produced in the final as in the initial phase. It is suggested to account for this large discrepancy that some other sources of electricity than that associated with membrane repolarization are present during contraction or relaxation of the muscle. The duration of the potential change coincides approximately with that of the contraction and relaxation of the muscle, and the "T phase" of cardiac muscle is incorporated within the final phase of the diphasic potential curve of the skeletal muscle, when this curve is recorded from a conducting field surrounding the muscle.

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[5] The relative simplicity of the whole curve is indicated by the fact that it can be closely approximated by a Fourier series of only two variable terms. Furthermore, the amplitude of both the initial and final phases varies with the sine of the angle that the axis along which the potential is recorded makes with the axis bisecting the muscle transversely. The curves along any axis are closely approximated by an equation of the form $y = \sin \Theta[0.56 + 1.023 \cos (t\text{-}24.8°) + 0.192 \cos 2t]$. The few terms necessary to obtain an approximation suggest perhaps that the fundamental physical changes underlying the formation of the curve are relatively few in number.

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INHIBITION OF THE HEART UNDER ANAEROBIC CONDITIONS[1]

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Attention has been directed in previous communications to the fact that vagus inhibition of the resting auricles of the turtle heart reduces the oxygen consumption much below the resting level (Garrey and Boykin, 1933, 1934, 1935). It was intimated that the reduced respiratory metabolism is a cognate phenomenon but is not an essential feature of the inhibitory mechanism. The present report gives evidence substantiating this statement, based on the study of cardiac inhibition under conditions which reduce respiratory metabolism.

PROCEDURE. The studies were conducted on the hearts of several species of turtle, chiefly Chelydra serpentina, Pseudemys elegans and Chrysemys picta. The sinus-auricle preparation with functioning vagi previously described by us (1934) served as the experimental object. The ventricle was cut away, the exsanguinated preparation removed from the body and thoroughly washed in bicarbonate buffered Ringer's solution (pH 7.6) for a half-hour or more. The auricles were mounted on an L-shaped glass-tube support and attached to a lever so that the entire contraction of both auricles could be recorded by the suspension method. The preparation could be surrounded by a moist chamber or immersed in fluid and gas led in as desired.

Effects of nitrogen. The anaerobic state was induced in the first instance by displacing the air in the moist chamber with a vigorous stream of nitrogen after removing all but the faintest traces of oxygen by bubbling it through a bead tower containing a solution of sodium hydrosulfite (Van Slyke and Neill, 1924) and then washing through Ringer's solution. It was found more convenient to immerse the preparation in boiled bicarbonate-buffered Ringer's solution (pH 7.6) and to keep a stream of scrubbed nitrogen bubbling through the solution. Under both the above conditions contractions will continue for at least twenty minutes, gradually weakening to extinction—a condition from which reversible recovery takes place

[1] This investigation was aided by a fluid research fund granted by the Rockefeller Foundation.

upon readmission of oxygen. The details of the degradation of contractions have been significantly discussed by Edsall, Hunt, Read and Redfield (1932) who have calculated that all available oxygen is used within five minutes, that the weakening of the contractions is due to the accumulation of metabolites, chiefly lactic acid, and that in a well buffered Ringer's solution quite strong contractions will continue for long periods. The observed weakening of the contractions is akin to fatigue and has nothing in common with true inhibition. We are justified in the conclusion that neither the reduction of oxidation *per se* nor the accompanying accumulation of lactic acid caused any condition remotely resembling the inhibitory state produced by vagus stimulation.

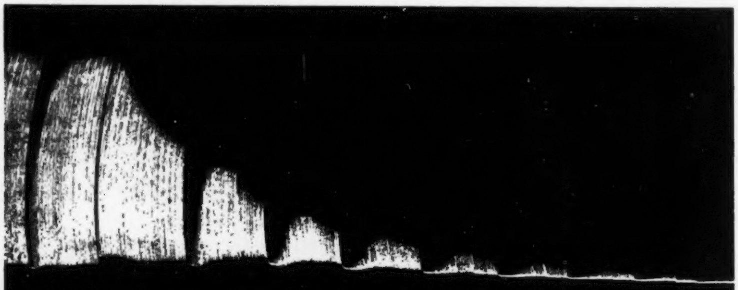

Fig. 1. Inhibition of turtle auricles in the anaerobic state. Vagus stimulation for ten second periods, repeated every five minutes, designated by figures indicating afternoon time, inhibited contractions, even the weakest. Normal effects at 1:30. Preparation placed in oxygen-free Ringer's solution at 1:35—(marked by arrow). Recovery (not shown) when oxygen was supplied was complete in one hour with vagus effects still demonstrable.

By vagus stimulation it was found that sinus and auricles beating under the above anaerobic conditions, could be inhibited, with suppression of rhythm, excitability and contractility. These effects could be induced at any stage of the anaerobic degradation, so long as the faintest trace of contraction was discernible, as well as during the stages of recovery which ensued when oxygen was again admitted to the preparation. An example of these inhibitory effects is shown in figure 1. Identical results were obtained with heart preparations immersed under mineral oil which had previously been heated to drive out all oxygen; they need no separate consideration. These experiments indicate unequivocally that the inhibitory mechanism is independent of oxygen supply and can operate even when there has been a marked accumulation of lactate in the cardiac muscle

such as takes place when the preparation is surrounded by an atmosphere of nitrogen, by unbuffered Ringer's solution or by mineral oil.

Cyanide experiments. As a check on the above anaerobic inhibition, preparations were subjected to the action of potassium cyanide which reduces respiratory metabolism. The literature of this subject failed to furnish desired data showing the quantitative effects of different concentrations of cyanide (M/100 to M/5000) in reducing the oxygen consumption by turtle auricles under the conditions of our experiments. Numerous determinations were therefore made by placing the resting auricular preparation previously described by us (1934) in Ringer's solution in the Barcroft-Warburg micro-respiration apparatus. The resting oxygen consumption thus determined was used for comparison with the oxygen consumption subsequently determined in a desired concentration of KCN in Ringer's solution. Details are unnecessary; a summary of the results is given in table 1. It is shown that a cyanide solution as strong as M/100

TABLE 1

Reduction of respiratory metabolism of resting turtle auricles with different concentrations of KCN in Ringer's solution, bicarbonate buffered, pH 7.5

Results expressed in percentage reduction as found in twenty-four experiments

	M/100	M/150	M/300	M/1000	M/5000
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Maximum	74	59.5	52.8	29.3	20
Minimum	65	53.0	45.6	24.9	6
Average of all experiments	68	55.7	49.6	27.8	11.3

reduces the oxygen consumption to about one-third the normal but that under the conditions existing in our experiments, oxidations are not completely suppressed. The effects of the cyanide upon oxygen consumption were discernible immediately upon immersion of the preparation in the cyanide solutions. A steady state of respiratory metabolism was initiated within five minutes and continued at a perfectly uniform rate for as much as three hours—longer runs were not made. The concentrations used were within the range of those desired for subsequent physiological experimentation with vagus inhibition.

When beating sinus-auricle preparations were immersed in buffered Ringer-cyanide solutions the tracings, like those under anaerobic conditions, show a rapid progressive weakening of the beats. The time required for suppression of contraction varies with the concentration of the cyanide; in M/300 cyanide, which reduces the oxygen consumption by fifty per cent, it may require as much as half an hour before the beats completely fail; when they do fail the cardiac muscle cannot be excited to contraction

by any stimulus although complete recovery ensues when the cyanide is washed away with oxygenated Ringer's solution.

It should be stressed in this connection that, in sharp contrast to the behavior of the beating preparation, auricles which had been brought to rest by careful excision of all of the sinus pacemaker could be soaked for more than two hours in M/100 KCN in Ringer's solution without losing their excitability or failing to contract when stimulated. It should be emphasized that this immunity from loss of physiological responsiveness in cyanide solutions for so long as two hours depends upon the maintenance of a state of rest, and apparently is attributable to the less rapid accumulation of metabolites (lactic acid) during rest than when contracting. The significant point attached to this finding is the fact that even prolonged depression of respiratory metabolism due to cyanide does not cause inhibition of the resting heart, since a non-excitable state is not thereby induced.

Vagus stimulation of a preparation under the influence even of strong cyanide concentrations, e.g., M/30, showed that inhibition can be induced at any stage of cyanide action, and as effectively as in the normal preparation. The results were entirely comparable to those obtained with the preparations in the anaerobic state which have already been described, and confirm the conclusions based on them. One added fact came to notice in certain preparations in which spontaneous sino-auricular block existed. As noted above, such auricles owing to their resting state, retain their excitability for a long time (hours) in cyanide solutions but it was found that vagus stimulation inhibited these resting auricles which thus became inexcitable during vagus stimulation.

From the foregoing description of inhibition in the anaerobic state it is evident that neither lack of oxygen nor lack of oxidations *per se* will cause inhibition, nor does the physiological state of auricular tissue induced by such depression of respiratory metabolism interfere with the induction of vagus inhibition. Among the consequences of lack of cellular oxidations is the accumulation of lactic acid and we have instituted inquiry to determine whether this substance, so intimately related to the energy requirements of repeated contractions, is in any way involved in the mechanism of vagus inhibition. To this end the tissues have been subjected to the action of monoiodoacetic acid which prevents the formation of lactic acid (Lundsgaard, 1930).

Monoiodoacetic acid experiments. Beating sinus-auricle preparations with functioning vagi were immersed in various concentrations of monoiodoacetic acid in Ringer's solution and the pH adjusted to 7.4–7.6. The effects upon the contractile processes and effectiveness of the vagi were studied. The concentration of 40 mgm. per cent was the strongest used and its effects will serve for illustrative description. There is an immediate

decrease in the height of the contractions which progressively weaken to about half their original height within fifteen minutes or half an hour—usually without any change in rate of rhythm. There is further progressive weakening of the contractions and after a variable time s/a blocks may develop irregularly and disturb the rhythm. Depending upon the condition of the individual preparation, sometimes within an hour, sometimes only after two or three hours, a tonic contracture sets in with progressive weakening and slowing of rhythmic contractions superimposed on the heightened base line. It may take two to four hours for maximum shortening to develop and for death in the state of alkaline rigor described by Lundsgaard to occur. Throughout this sequence of events vagus stimula-

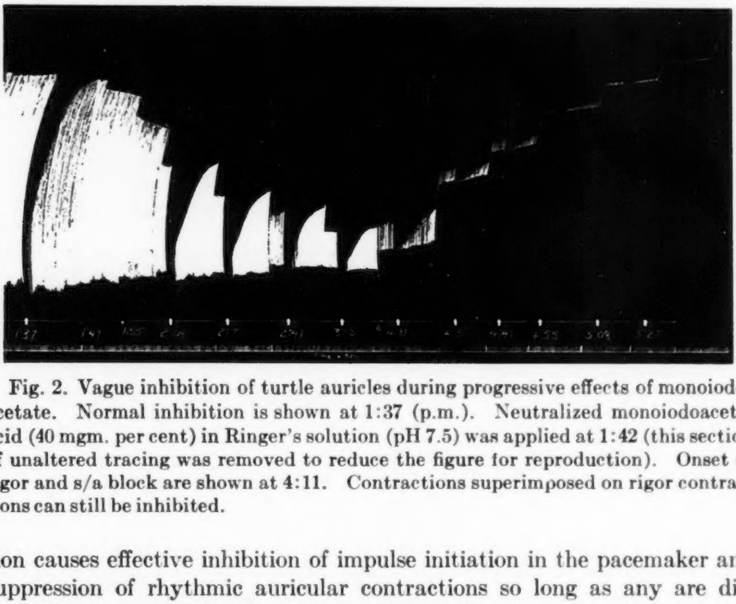

Fig. 2. Vague inhibition of turtle auricles during progressive effects of monoiodoacetate. Normal inhibition is shown at 1:37 (p.m.). Neutralized monoiodoacetic acid (40 mgm. per cent) in Ringer's solution (pH 7.5) was applied at 1:42 (this section of unaltered tracing was removed to reduce the figure for reproduction). Onset of rigor and s/a block are shown at 4:11. Contractions superimposed on rigor contractions can still be inhibited.

tion causes effective inhibition of impulse initiation in the pacemaker and suppression of rhythmic auricular contractions so long as any are distinguishable, even when they are superimposed on the curve of contracture as rigor progresses. An illustration to these effects is shown in figure 2. The experiments with monoiodoacetic acid justify the further conclusion that vagus inhibition of the turtle's heart is not dependent upon either the production or accumulation of lactic acid. Similarly the degradation of contractions cannot be due to lactic acid and probably not to the accumulation of other metabolites. The possibility of exhaustion of phosphocreatine supplies is to be thought of, but the progressive onset of rigor indicates a more profound physico-chemical toxicity.

SUMMARY

Reduction of respiratory metabolism of turtle auricles, e.g., by an atmosphere of nitrogen or in cyanide solutions, or after treatment with monoiodoacetic acid, does not cause inhibition of rhythmic contractions. The reduction of oxygen consumption which has been found to accompany vagus inhibition is not an essential feature of the inhibitory process. Vagus inhibition is not interfered with either by preventing oxidations or by absence of lactic acid due to treatment with monoiodoacetic acid; if cardiac muscle can contract it can be inhibited, provided it has an inhibitory nerve supply which functions.

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LOCAL VARIATIONS IN THE NORMAL POLYNUCLEAR COUNT IN MAN

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The recent study by Kennedy (1933) on the normal polynuclear count in man concludes that the "health standard" laid down by Cooke and Ponder (1927) is substantially correct. The figures as originally given by Cooke are:

	I	II	III	IV	V	*Mean*
Lowest normal count	15	34	40	11	0	2.47
Average normal count	12	25	44	15	4	2.74
Highest normal count	9	24	47	17	3	3.11

These figures are based on the counts of 90 normal persons of both sexes between the ages of 12 and 55, after the most rigorous physical examination to eliminate the possibility of even minor infections. The figures given by Kennedy are also based upon the counts for 90 young adults of both sexes, although the "health standard" is one of relative laxity compared to that of Cooke. Kennedy's figures are:

	I	II	III	IV	V	*Mean*
Lowest normal count	30	37	18	14.5	0.5	2.175
Average normal count	13	30	43	10	1	2.62
Highest normal count	10.5	22	39.5	28	4	3.05

Kennedy concludes that the slightly lower value of the mean of the average normal count is due to the relative laxity of his health standard, and that the range of values for "normal" polynuclear counts, given by Cooke and Ponder, are not so narrow as to exclude the ordinarily "healthy" individual.

Several months ago Abels (1934) made a study of the normal count in healthy individuals in New York and obtained figures which differ radically from those of Cooke and Ponder and of Kennedy. Abels' figures are:

	I	II	III	IV	V	*Mean*
Lowest normal count	15	59	24	1	1	2.14
Average normal count	12	54	27	6	1	2.30
Highest normal count	9	40	38	12	1	2.56

These values were found for 100 persons of both sexes "who, to the best of their knowledge, had suffered from no acute or chronic infection for several months, and whom the clinician and physiologist would ordinarily consider normal." It is obvious that Abels' figures are incompatible with those of Cooke and Ponder and of Kennedy. As pointed out by Abels, there are three possible explanations for the different results. 1. Cooke's "normal count" may be obtained only in persons conforming to the most rigorous of "health standards" and Kennedy, using a less rigorous "health standard," may have obtained nearly coincident results by "counting high" (i.e., applying Cooke's criterion of nuclear lobulation too loosely). 2. Abels may have "counted low" (i.e., applied Cooke's criterion too strictly), but he took the precaution of having his counts checked by Ponder and by myself. 3. The average polynuclear count may differ according to locality, race, climate, etc., and a considerable amount of evidence in the literature leads one to suspect that this may be so. MacFie (1915) for instance, says that the Arneth count (by Arneth's original method) of normal white people in South Africa is more left-handed than that of normal Europeans and more nearly like that of the normal native. His figures, however, are far from convincing. Breinl and Priestley's (1917) Arneth counts made in North Australia and New Guinea show that the normal adult mean for white people in these regions is about 2.0 and not greatly different from that of the native, but much lower than the value 2.75 given by Arneth for the normal European, and Bannerjee (1923–24) gives a mean of 2.42 for the normal Bengalee, which is less than Arneth's mean for Europeans.

It is extremely difficult to assess the value of these investigations, for Arneth's method of counting, which gives very variable results in the hands of different persons, was used in every case, and there is no evidence that any of the investigators would themselves have obtained Arneth's mean of 2.75 for normal people in Europe. The differences recorded may therefore have been due to the method of counting. At the same time there is very definite evidence that there are differences in the average polynuclear counts of certain kinds of animals, the differences apparently depending either on the genetic strain or the locality. Thus Kennedy and Climenko (1931) give 2.35 as the mean for rats, while Yeager and Haterius (1930) give 1.09, and Vaughan and Gunn (1930) give an even lower figure. Cooke's criterion was used in both Kennedy and Climenko's investigation (Scotland) and in Yeager and Haterius' (New York) and the difference is far too great to be accounted for by errors in counting. Kennedy and Climenko give 2.52 as the mean for normal sheep and 2.34 as the mean for normal cattle (Scotland); Ethel D. Simpson, however, gives 1.24 for sheep and 1.28 for normal cattle (Ithaca, New York). Again the discrepancy is too great to be accounted for by differences in the criteria used for count-

ing, and Miss Simpson's results are confirmed by Sergent and his collaborators working in North Africa (Simpson 1929, Sergent et al.; 1929).

While these results indicate that genetic or geographical differences may exist in the case of animals, no systematic investigation has been made in the case of man. This study was begun with the object of obtaining blood films from widely scattered localities, staining them uniformly, and mak-

TABLE 1

	MEAN	HIGH	LOW	σ	S.E. OF MEAN
Australia	2.64	2.96	2.18	0.19	±0.038
Wigan (England)	2.61	2.99	2.19	0.20	±0.04
Florida	2.58	2.87	2.24	0.16	±0.032
California	2.57	2.84	2.23	0.17	±0.034
Alberta	2.55	2.94	2.13	0.23	±0.046
Colorado	2.45	2.93	1.90	0.22	±0.046
South Africa	2.44	2.86	1.67	0.25	±0.05
New York	2.44	2.78	2.18	0.16	±0.032
Japan	2.34	2.86	1.86	0.20	±0.043
China	2.33	2.61	2.02	0.22	±0.033
Greece	2.31	2.79	1.78	0.27	±0.06

TABLE 2

	AUSTRALIA	WIGAN	FLORIDA	CALIFORNIA	ALBERTA	COLORADO	SOUTH AFRICA	NEW YORK	JAPAN	CHINA	GREECE
Australia		0.5	1.2	1.4	1.5	3.16	3.33	4.0	5.36	6.2	4.7
Wigan (England)	0.5		0.6	0.77	1.0	3.1	3.07	3.9	5.0	5.38	4.6
Florida	1.2	0.6		0.22	0.53	2.32	2.4	3.1	4.6	5.43	4.0
California	1.4	0.77	0.22		0.35	2.1	2.1	3.0	4.2	5.1	3.7
Alberta	1.5	1.0	0.53	0.35		1.5	1.6	2.0	3.3	3.88	3.2
Colorado	3.16	3.1	2.32	2.1	1.5		0.15	0.18	1.7	2.1	1.8
South Africa	3.33	3.07	2.4	2.1	1.6	0.15		0.00	1.5	1.8	1.6
New York	4.0	3.9	3.1	3.0	2.0	0.18	0.00		1.8	2.4	1.9
Japan	5.36	5.0	4.6	4.2	3.3	1.7	1.5	1.8		0.20	0.40
China	6.2	5.38	5.43	5.1	3.88	2.1	1.8	2.4	0.20		0.30
Greece	4.7	4.6	4.0	3.7	3.2	1.8	1.6	1.9	0.4	0.30	

ing polynuclear counts, using the same criterion of nuclear configuration throughout. This procedure eliminates one of the principal sources of error, i.e., varying conceptions of what constitutes a nuclear lobe. Blood films from "normal" adults (i.e., adults who, to the best of their own knowledge, were in good health and whom the physiologist or clinician would ordinarily consider normal) were accordingly obtained from universities

and hospitals in various localities. Twenty-five unstained, unfixed blood-films were received from each center and 100 cells counted in each film, Wright's stain being used in all cases and the Cooke and Ponder criterion used throughout. Differential counts were not made but certain slides from Japan and Greece were rejected because of definite indications of abnormality in the distribution of the other classes of white cells. Tables 1 and 2 show the results.

It will be seen that the means for the various countries vary considerably, the highest mean being obtained from Australia and the lowest from Greece. Table 2 shows the number of times the S.E. of the differences between pairs of means exceeds its own S.E. Considered in this fashion, the means for Australia, Wigan, Florida, California, and Alberta do not differ significantly. The means from New York and Colorado, however, are significantly different from those of the above localities, and are in the same class as those of South Africa, Japan, China, and Greece.

Discussion. These results go far to reconcile existing differences of opinion regarding the figures for the "normal" polynuclear count, for it seems that the occurrence of the different values can be attributed to differences of locality instead of to different methods of counting, etc., as has been the tendency. Thus, as Abels says, the high mean of Cooke and Ponder is not found for a random group of "normals" in New York, but it is found for a similarly selected group in Wigan, England (cf. Cooke), and in Edinburgh, Scotland (cf. Kennedy), and also in several other localities, e.g., California and Florida. This paper is not concerned with the possible explanations for the differences, for too many factors require to be considered, and the temptation exists to accept any one of them as a ready explanation. Climatic conditions, at least, in the ordinary sense of the word, do not appear to be a factor of much importance, for the climate is not uniformly better in those localities associated with a high count than in those associated with a low count.

One of the most important points, however, is that for normal subjects from *the same* locality, the polynuclear mean is remarkably constant. This means that the polynuclear count is a very sensitive index of normality in the particular conditions under which that normality exists, and that errors and differences in the criteria used for counting, far from being so great as to produce the variations observed, are actually so small that the real differences are readily detected when they occur.

SUMMARY

Figures are given for polynuclear counts made on blood films obtained from various geographical centers.

These figures indicate that definite differences exist between the means of various localities though the mean for each locality is quite constant.

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FUNCTIONAL STUDIES OF THE NERVOUS SYSTEM IN EXPERIMENTAL BERIBERI[1]

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The characteristic manifestations of beriberi or "polyneuritis" resulting from lack of vitamin $B(B_1)$[2] are cessation of growth with loss of appetite, and functional disturbances of the nervous system leading to disability and death. Many neurologic aberrations have been attributed to vitamin $B(B_1)$ deficiency, including paralysis, convulsions, spasticity, flaccidity, ataxia and incoördination. Recent investigators have questioned the interpretation of some of these symptoms in experimental animals, as well as their correlation with lack of vitamin $B(B_1)$. The study here presented was undertaken to define the syndrome resulting from lack of the heat-labile vitamin B of yeast and to ascertain the nature and extent of the functional disturbances of the nervous system in this deficiency.

The significant alteration in vestibular function, as shown by the duration of nystagmus following a standardized rotational stimulus, has been described in a preliminary report (Church, 1933). Nystagmus duration increases progressively with deficiency in vitamin $B(B_1)$, reaching a value often 2 or 3 times the normal before the appearance of other neurologic symptoms.

REVIEW. Woollard (1927) found no evidence of paralysis in polyneuritic pigeons and pointed out that head retraction depends not on changes in the nerves of the neck muscles but on alterations in muscle tone. Mouriquand, Leulier and Morin (1933) at no stage observed paralysis in the strict sense of the term in rice-fed pigeons. The disturbances of flight, locomotion and posture in these pigeons, they state, resembled ataxia rather than paralysis. Grinker and Kandel (1933) note that some of their rats on B_1 deficient diet dragged their hind legs, but that in no case was

[1] These studies were carried on under the Mead Johnson Fellowship in Pediatrics at the University of Pennsylvania.

[2] Vitamin $B(B_1)$ refers to the heat-labile fraction and vitamin $G(B_2)$ to the heat-stable fraction of the vitamin B complex.

there true paralysis. Convulsions, according to Amantea (1928), are not a characteristic feature of beriberi in pigeons. He noted that when the bird is quieted it maintains its normal posture, but when disturbed enters into a violent excess of agitation.

The reflexes of beriberi rats have been examined by Woollard (1927, 1932), who found that all the righting reflexes were present and the deep reflexes were normal. He also states that the animals were not spastic. On the other hand, Prickett (1934) reports extreme spasticity in later stages of vitamin $B(B_1)$ deficiency in rats.

Drury, Harris and Maudsley (1930) made an electrocardiographic study of vitamin B deficiency in the rat. They found bradycardia to be a distinctive feature, the heart rate being reduced from the normal rate of 500 to 525 beats per minute to 300 or 350 per minute. The bradycardia was of sinus origin and not due to vagal influence.

Measurements of chronaxie in rice-fed pigeons were reported by Mouriquand, Leulier and Morin (1933). Before the appearance of the convulsive crises chronaxie varied slightly, showing no significant increase indicative of lesions of the peripheral motor neurone. During the crises or in the hours preceding there were changes in chronaxie in various groups of muscles conforming to the unequal distribution of tonus. This was particularly marked in the muscles of the neck. They conclude that the disturbances were functional since they were rapidly reversible.

Experimental technic. The symptoms of beriberi were regularly produced in white rats of Wistar strain by feeding one of the experimental diets (table 1) supplemented with cod liver oil and autoclaved yeast. Control animals from each litter were given the same diet and cod liver oil, with whole dried yeast in place of the autoclaved material. The number of animals used in these experiments was 272, of which 94 were controls. Many of the control animals were later made deficient to permit the study of the beriberi syndrome in adult as well as in young growing animals.

Each rat was confined to an individual cage with wide-mesh screen bottom. Food was given in weighed portions either daily or as needed, the excess being weighed back semi-weekly. Supplements were fed separately in castor cups, this method permitting the administration of uniform daily doses and a careful check upon their consumption. Each animal was weighed and examined daily.

One to three litters of young rats were used in each experiment. They were placed in individual cages at 23 to 28 days of age and were given the experimental diet supplemented with cod liver oil and whole yeast. After a preliminary period on adequate vitamin B, to accustom the animals to their new diet and surroundings and to establish the normal rate of growth on this diet, autoclaved yeast was substituted for whole yeast in the supplement, all other factors remaining unchanged. Control animals from each

litter were continued on the same diet and whole yeast. The effect of withdrawal of the heat-labile vitamin B of yeast was thus doubly demonstrated by *a*, comparison of the same animal before and after substitution of autoclaved for whole yeast, and *b*, comparison of the deficient animal with the control.

Paired feeding. In order to rule out the effects of inanition resulting from the spontaneous restriction of food intake in beriberi the paired feeding technic (Mitchell, 1933) was employed in 4 experiments (42 animals). In this way the intakes of the two rats were equalized, the only difference between them being that one received autoclaved yeast and the other whole yeast. Thus, any symptoms due to inanition could be differentiated from those resulting specifically from lack of vitamin $B(B_1)$.

TABLE 1

Experimental diets

	DIET 31	DIET 31A	DIET 35
	grams	*grams*	*grams*
Casein (vitamin A and B free)*	16	16	18
Sucrose	58	78	66
Lard**	20	—	10
Salt mixture†	4	4	4
Agar	2	2	2

Daily supplementary feeding:
- Cod liver oil .... 2 drops
- Autoclaved yeast‡ .... 0.54 gram

* Extracted with cold 60 per cent alcohol and hot 95 per cent alcohol.

** Armour's Star Brand, heated above melting point before mixing with diet.

† Osborne and Mendel.

‡ Dried brewers' yeast from Northwestern Yeast Company, autoclaved in shallow pans at 120° and 16 pounds pressure for three hours.

I. THE BERIBERI SYNDROME IN THE RAT. The earliest characteristic evidence of vitamin $B(B_1)$ deficiency was the decline in growth rate accompanied by voluntary restriction of food intake. Growth ceased in 7 to 14 days following withdrawal of vitamin $B(B_1)$ from the diet, and food consumption declined progressively after the first week. Since these early changes in beriberi have no known neurologic basis they will not be discussed further in this paper.

Certain symptoms developed as a result of inanition in the restricted controls receiving adequate vitamin B in paired feeding experiments, as well as in the deficient animals. These symptoms should be excluded in discussing the effects of lack of vitamin $B(B_1)$ itself, and are therefore mentioned at this time. Priapism developed in the males of the restricted

control group as well as in the deficient males. All the animals became emaciated and in several instances the control on restricted food intake died at about the same time as the deficient rat. Two of these controls showed convulsive phenomena suggestive of the neuromuscular symptoms of beriberi, but these convulsive seizures were strictly terminal events, differing, as will be discussed later, from the disturbances characteristic of vitamin $B(B_1)$ deficiency.

During the first 3 or 4 weeks on deficient diet the only symptoms characteristic of lack of vitamin $B(B_1)$ were failure of growth and diminishing appetite. Fluid intake was likewise diminished. Inanition was responsible, as already mentioned, for certain other symptoms which occurred even with adequate intake of vitamin $B(B_1)$. Manifestations of neurologic involvement made their appearance usually in the 4th or 5th week. These neuromuscular symptoms conformed in general to the following pattern: 1, changes in muscular tonus; 2, ataxia; 3, disturbances of equilibrium; and 4, increased excitability. Because of the conflicting interpretations previously mentioned, these symptoms will be described in detail.

1. Changes in muscular tonus. The earliest neuromuscular symptom of beriberi was usually marked by transitory diminution in muscle tone, readily detected on taking the animal in the hand. The rat felt extremely limp and was incapable of making quick postural adjustments, as when the hand was suddenly inclined. This hypotonic condition often lasted only 1 or 2 days, normal tone being frequently regained before the onset of ataxia. Occasionally, instead of being diminished, muscular tonus was increased during this stage. The significance of these observations will be discussed later.

2. Ataxia. The primary alteration in muscular tonus was followed in 2 or 3 days by disturbances of muscular coördination, which rapidly developed into the acute stage of beriberi. Irregularity of posture was the first evidence of this lack of coördination. The hind legs assumed ungainly attitudes, which the animal made no effort to correct. The gait became asynergic, movements of locomotion being jerky and uncoördinated. When the ataxic animal was upset upon the table it regained its footing quickly but clumsily.

3. Disturbances of equilibrium. After 1 or 2 days of increasing ataxia, disturbances of equilibrium made their appearance. These were easily demonstrated by means of the "upsetting test," the animal being placed upon its back upon the table and released. The normal result is an immediate return to standing posture. At this stage in beriberi, however, the animal struggled intensely but inefficiently to regain its footing, kicking and twisting and whirling its tail until this was achieved. The body was then braced against any object which offered support, or flattened to the table with legs outspread, in an effort to gain greater stability of posture.

On coming near the edge of the table the animal went over without the slightest hesitation, resuming its struggles when caught in the hand.

That these struggles are the consequence of a disturbance of equilibrium is further evidenced by the fact that they cease when the animal is supported in the normal standing position. When freed after a few moments of quiet support in the hand it often walks off without difficulty except for its ataxic gait. This procedure has been repeated time after time. Upsetting the animal brings on the evidences of disturbed sense of equilibrium and leads to agitated endeavors to regain standing posture, the struggles ceasing when this is achieved (see fig. 1).

The disturbance of equilibrium is sometimes symmetric, the animal falling to either side, and, in recovering, overcorrecting and falling to the other side; or the head may be retracted, the animal tending to fall backward. Asymmetric disturbance is more common, however: the head is tilted to one side, the body is curved, and the legs are extended in abduction on the side toward which the head (chin) is tilted, the opposite legs being partially flexed. The body is sometimes twisted, with the head as the leading segment. Such an animal, when upset, often rolls over and over in remarkably swift revolutions until progress is impeded. Circling in the horizontal plane is another manifestation of disturbed sense of balance. The animal may pivot on the hind quarters and circle about in a series of falling movements, rising on its fore-legs only to lose its balance and fall again in the same direction.

Spontaneous nystagmus has been observed occasionally during this stage, the eye movements being of various types, lateral, vertical and rotatory. Usually, however, the quick component is lacking, the eyes remaining deviated, or both components are absent, the eyes being fixed and immobile. Marked and often unequal dilatation of the pupils may accompany the agitated struggles, returning to normal when the animal is quieted. Varying degrees of exophthalmos have been noted.

4. Hyperexcitability. The beriberi animal becomes more and more easily disequilibrated and wears itself out with struggles to recover and maintain standing posture. Concurrently, nervous excitability increases until a slight touch or even a loud noise will "upset" it sufficiently to bring on the agitated endeavors to regain equilibrium. Rapid kicking movements are sometimes exhibited, giving way in the stage of exhaustion to generalized tremors resulting from poorly sustained muscular effort. True convulsions, as discussed below, are seldom, if ever, present. The combination of ataxia, disturbed equilibratory sense, and excitability often results in the animal grasping and clinging tenaciously to any object within reach, even its own tail or leg.

These struggles in response to slight stimuli cease only when the animal is exhausted and are often resumed after rest. Since little or no food is

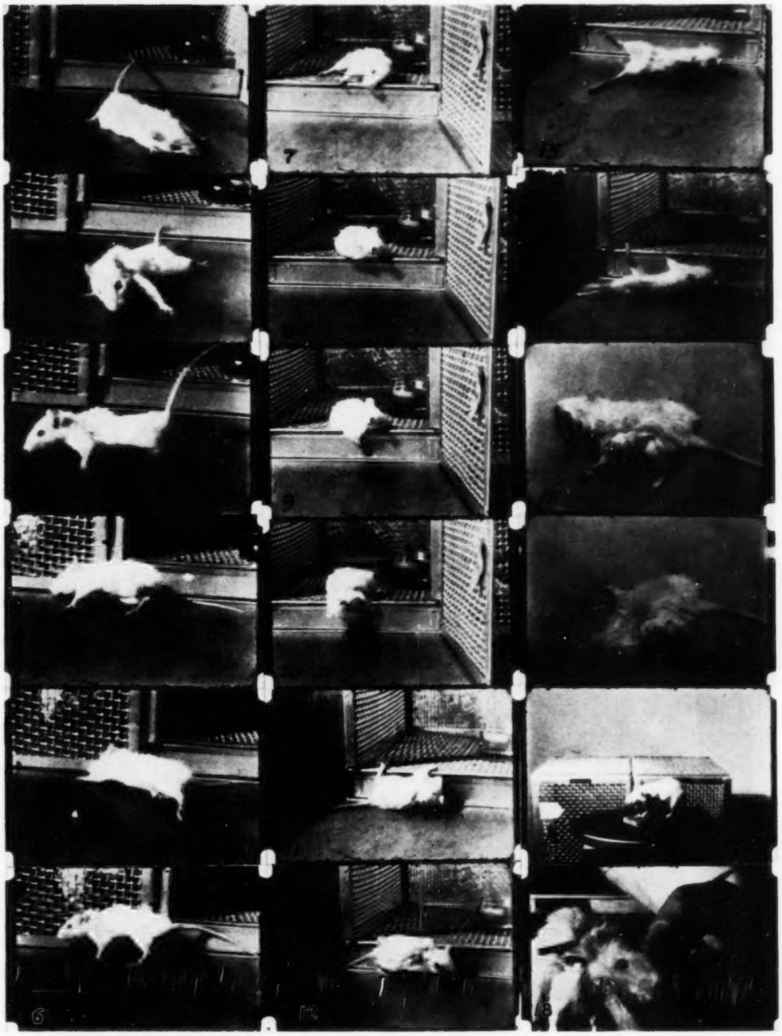

Fig. 1. Experimental beriberi in the rat. (1–6) Cinematograph sequences showing disturbance of equilibrium and struggle to regain standing posture. (7–12) Ataxia and loss of equilibrium sense. (13–14) Rapid kicking movements in struggle to regain footing. True convulsions are absent. (15–16) Asymmetric posture (viewed from directly above). (17–18) The vestibular function test applied to the rat.

eaten at this stage there is rapid loss of weight, the animal gradually becomes hypothermic and lethargic, and death ensues 2 or 3 days after the onset of vertiginous symptoms.

Other symptoms. The agitated struggles of the beriberi rat to recover equilibrium often resemble true convulsions but may be differentiated in that they are *a*, voluntarily initiated; *b*, purposive but uncoördinated, and that *c*, they cease when the animal is supported in normal posture. True convulsions have been observed in the terminal stage shortly before death, and have occurred in controls on restricted intake as well as in deficient animals. Tremors resulting from poorly sustained muscular effort are readily distinguished from convulsions.

An occasional animal in the acute stage has shown marked hypertonicity. The legs were rigidly extended and any attempt on the part of the observer to produce flexion by pressing against the (hind) foot resulted instead in rotation of the pelvis. In several instances this hypertonicity was so exaggerated in the antigravity muscles as to simulate the posture often assumed in decerebrate rigidity. The animal, when placed upon its feet, stood stiffly with neck retracted, back arched and tail elevated. The crossed extensor reflex has been obtained in such an animal, further proof that the nervous system, so far as postural reflexes were concerned, was functionally that of a decorticate animal.

Except for muscular hypotonia or rigidity there has been no evidence of loss of motor function in beriberi prior to the terminal stage. The animal, even when rigid or extremely limp, responded to painful stimulation by withdrawal of the member stimulated, proving the essential integrity of the sensory and motor nerves and the spinal reflex arc. Paralysis in the strict sense is therefore not a feature of beriberi in the rat. The only symptom which may result from a peripheral loss of sensation is ataxia, and a further study of the functional integrity of the proprioceptive fibers and end-organs in muscle and tendon was deemed necessary in order to answer this question. The results of this study are reported in section II.

Reflexes: Righting reflexes. The labyrinthine righting reflex resulting in the normal tendency of the rat to face upward and to climb (negative geotropism) was tested by means of a tilting screen, the meshes of which afforded a satisfactory foothold for the animal. The deficient rat prior to the appearance of the disturbances of equilibrium almost invariably showed negative geotropism, as did the normal controls. With the onset of these symptoms, however, the animal became indifferent to its position with respect to the earth, showing no tendency to orient itself upon the screen. This failure to exhibit the labyrinthine righting reflex occurred even when the animal was unexcited and moving about upon the screen without difficulty. Neck and body righting reflexes, on the other hand, were not impaired. When the animal was supported horizontally in mid-air by head and tail the body could be rotated at will, using the head as a "handle."

This again indicates loss of the labyrinthine reflex, but preservation of neck righting reflexes. When the body or limbs were brought in contact with any object, the body righting reflex was evidenced in the attempt to assume a posture normal to that surface.

The animals used in this study were tested frequently for "hopping," "landing" and "placing" reflexes. The hopping and landing reflexes were generally elicited in both beriberi and control animals, while the placing reflex was often not obtainable in either.

Deep reflexes. The response of the normal rat to lightly tapping the knee or tibia is a reflex contraction of the tibialis muscle, causing dorsiflexion of the foot. This reflex was found usually intact in vitamin-B(B_1)-deficient animals. The same stimulus, moreover, often produced in these deficient rats a dorsiflexion reflex of the contralateral foot; and in the acute stage of beriberi tapping the tibia frequently caused flexion of the tail. This movement of the tail was usually in the direction of the side stimulated and exhibited the characteristics of a true reflex. By fixing the tail near the base it was demonstrated that flexion occurred in the distal portion as well as at the base. This pathologic tail reflex, which has not to our knowledge been previously described, indicates spreading of the reflex arc in the spinal cord. Seventy-four per cent of the animals examined during the acute stage of beriberi exhibited this reflex.

DISCUSSION. The nervous disturbances resulting from lack of vitamin B(B_1) show many variations in detail in individual rats but conform to the pattern of a functional impairment of the proprioceptive system. This pattern consists in 1, changes in muscular tonus; 2, ataxia, and 3, disturbances of equilibrium. To this is usually added 4, hyperexcitability, a condition which possibly results from irritation of the nervous system by the same lesion which produces the proprioceptive disturbances. The nutritive state of the animal has considerable influence upon the character and degree of the symptoms manifested, emaciated and weakened rats often passing from hypotonia to terminal lethargy with only mild evidences of incoördination and disturbed equilibrium and without any increase in excitability. When the neurologic disturbances appear while the animal is still strong and not emaciated, remarkable exhibitions of uncoördinated and disequilibrated activity are given upon slight stimulation. The available supply of energy for muscular activity is doubtless a factor governing this difference in neuromuscular symptoms. It has been frequently noted by others as well as ourselves that partial or incomplete deficiency in vitamin B(B_1) is often attended by more remarkable evidences of neurologic disorder than is the complete absence of this vitamin. Nerve function, therefore, cannot be judged by the degree of deranged neuromuscular activity. Certain functional tests, however, are of value in determining the location and extent of the lesion responsible for these disturbances.

Loss of proprioceptive sense as shown by ataxia may result from a func-

tional impairment of the peripheral nervous system, the myelinated proprioceptive fibers of which terminate in neuromuscular and neurotendinous spindles and in Pacinian corpuscles (Ranson, 1927). In a careful histological study of the nerve endings in beriberi rats, Woollard (1927) found that they were frequently swollen with loss of their finer differentiation. He observes that the "degree of the changes in the intermuscular portion of the nerve fibers and the nerve endings as compared with the paucity or absence of the changes elsewhere suggests that the disease exerts its effect at this point." In order to test this hypothesis the study reported below was undertaken.

II. The functional integrity of proprioceptive end-organs and fibers.[3] When the intact muscle and tendon are stretched, either actively or passively, afferent impulses arising in the neuromuscular and neurotendinous spindles are transmitted to the central nervous system by way of the proprioceptive fibers in the nerve trunk. With the animal anesthetized, these nerve impulses can be led off by means of suitable electrodes and the nerve action potentials resulting from passive stretching of the muscle and tendon can be amplified and recorded by the method of Adrian (1932), Bronk (1929), and Matthews (1931). A loss of function in these neurologic elements in vitamin-B(B_1)-deficient animals should therefore be demonstrable by comparison of the photographic records of the nerve impulses with those obtained by the same technic in normal control animals. This method has been employed in 11 experiments comprising 8 deficient and 3 normal animals. Representative protocols and a summary of the results are given below.

Protocol 1—5/6/32. Rat 10B7—normal control 72 days old, on diet 31 with cod liver oil and whole yeast for 44 days. Weight 107 grams. Vestibular test (120 r.p.m.) gives nystagmus lasting 11 seconds. *Operation:* Injected 10 mgm. of sodium amytal intraperitoneally, the animal losing consciousness in about 1 minute. The nerve to the tibialis muscle was dissected out, care being taken to prevent injury to or drying of the nerve. The nerve was sectioned near the pelvis and the peripheral part was stimulated by an induction shock to prove its function. The tendon of the muscle was freed from its insertion in the tarsal bone after tying a thread securely about it. The cut end of the nerve with its peripheral endings intact was then mounted upon non-polarizable brush electrodes and connected with amplifier and oscillograph. When the muscle was stretched by pulling the thread attached to the tendon the nerve impulses were recorded photographically.

Protocol 7—6/27/32. Rat 13B6—vitamin-B(B_1)-deficient animal 72 days old, on diet 31 with cod liver oil and autoclaved yeast for 39 days. Weight 68 grams. Marked neuromuscular symptoms with ataxia and loss of equilibrium sense. Falls to either side in attempt to stand. Extremely hypertonic, posture at times suggesting that of decerebrate rigidity. Exophthalmos is marked and pupils are widely dilated. The vestibular test (120 r.p.m.) gives short rapid nystagmic movements

[3] This study was conducted in the Eldridge R. Johnson Foundation for Medical Physics at the University of Pennsylvania, under the supervision of Dr. D. W. Bronk.

lasting 16 seconds. *Operation:* Injected 5 mgm. of sodium amytal into the peritoneal cavity. The technic was the same as that given in the previous protocol. On stimulating the nerve there was a good responding twitch of the tibialis muscle. With the nerve mounted on the electrodes, stretching the muscle and tendon produced a burst of impulses, which were recorded as before. The animal was allowed to recover from the anesthesia, when the neuromuscular symptoms of beriberi were again exhibited and the vestibular test showed prolonged nystagmus (40 seconds).

Results of oscillograph experiments. In only one of the 11 animals did stretching the muscle and tendon fail to give a response. This rat (10B13), on deficient diet, gave no response on stretching the left tibialis muscle and tendon. However, the right tibialis and both gastrocnemii gave good responses, suggesting that an operative injury to the nerve was the cause for the lack of response of the left tibialis.

Comparison of the oscillograph records of the afferent impulses resulting from stretching the tibialis muscle and tendon of the deficient rats with those of the normal controls showed no significant differences. It is evident, therefore, that the functional integrity of at least the major proportion of the muscular and tendinous end-organs and their peripheral fibers is preserved in beriberi. The marked ataxia observed in this disease must therefore result from a lesion elsewhere in the nervous system.

III. Vestibular function. The functional disturbances of the proprioceptive system in beriberi were further investigated by the examination of vestibular function as manifested in the character and duration of nystagmus following rotation. The significant and progressive increase in nystagmus duration in animals deprived of the heat-labile fraction of yeast, together with the technic employed, has been reported in a preliminary note (Church, 1933). These experiments have been continued and the results are given here.

Relation of speed of rotation to duration of nystagmus. Normal rats were rotated to 30, 60, 90 and 120 revolutions per minute for periods varying from 2 seconds to 20 seconds, in order to discover the relation of speed and duration of rotation to the vestibular response. The duration of nystagmus following rotation at each speed is represented by the curves (fig. 2). It is apparent from these curves and repeatedly confirmed in other experiments that the duration of nystagmus is dependent upon the speed of rotation and the condition of the animal, and is independent of the period of rotation beyond a certain minimum period necessary to accustom the animal to the constant speed. The maximum duration of nystagmus following rotation at 60 r.p.m. is attained after 12 seconds, while at 120 r.p.m. it is necessary to rotate for 18 seconds or longer. Continued rotation does not appreciably alter the duration of nystagmus (Prince, 1919).

Vestibular function tests: first series (120 r.p.m.). The technic of these experiments was that already described in the preliminary report. In

figure 3 are given the observations of nystagmus duration in 2 rats over a period of 17 weeks, beginning when the animals were in the 9th week on the experimental diet. The rat on autoclaved yeast was given a single dose of vitamin B(B_1) concentrate[4] each time the neuromuscular symptoms of beriberi (NM) were exhibited, the remission being prompt but temporary. The duration of nystagmus in this animal, shown on the chart by the black dots, was consistently longer than that of the control, except for the drop following treatment. When the diets were interchanged, the previously deficient animal being given whole yeast and the control animal being changed to autoclaved yeast, the results of the vestibular tests show a gradual transposition. Nystagmus duration increased in the animal made deficient in vitamin B(B_1), and diminished to the normal range in

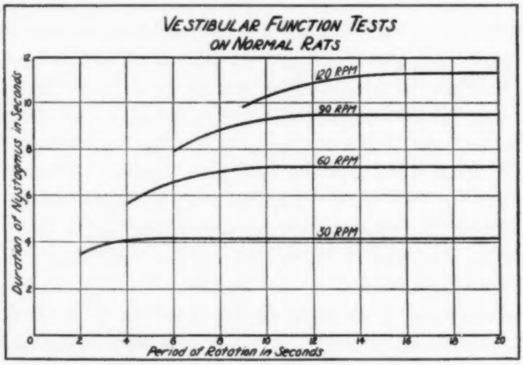

Fig. 2. Relation of speed and period of rotation to the vestibular response. Duration of nystagmus in normal animals is dependent upon rate of rotation and is independent of the period of rotation beyond a certain minimum period required to reach equilibrium at the constant speed.

the animal now supplied with this factor. The mean duration of nystagmus for each rat in the period before interchange and again after an interval of 4 weeks (depletion period) following interchange of the yeast supplements, is given below:

	Period I		*Period II*	
	Vitamin B(B_1)	*Nystagmus seconds*	*Vitamin B(B_1)*	*Nystagmus seconds*
Rat 307A................	Deficient	20.97 ± 0.36	Adequate	12.85 ± 0.28
Rat 307E................	Adequate	10.68 ± 0.19	Deficient	18.13 ± 0.69

[4] Prepared by Mead Johnson Company, Evansville, Ind., from rice polishings. This concentrate contained approximately 140 rat units per cubic centimeter. A 0.020 cc. dose administered by mouth sufficed to cure the symptoms of beriberi and prevent their recurrence for 5 to 9 days.

It should be noted that, while nystagmus was present and prolonged during the first acute neuromuscular attack (NM) in each rat, no nystagmus was elicited by rotation during subsequent recurrences of the symptoms. Following treatment with vitamin B(B_1) reflex nystagmus promptly returned and was prolonged. Loss of the vestibulo-ocular reflex thus appears to be a progressive rather than a regressive phenomenon in beriberi.

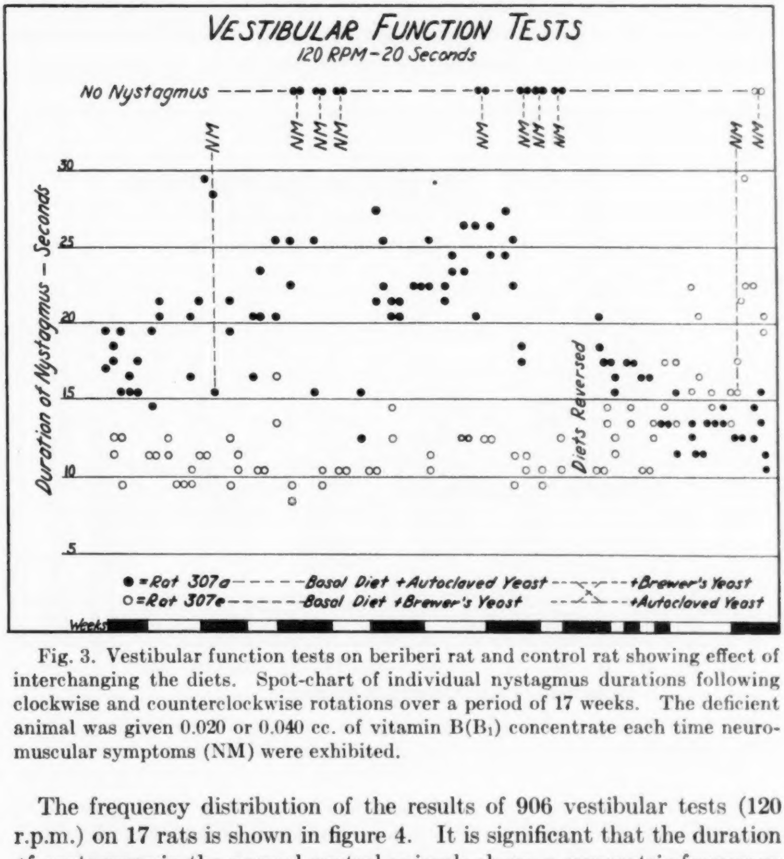

Fig. 3. Vestibular function tests on beriberi rat and control rat showing effect of interchanging the diets. Spot-chart of individual nystagmus durations following clockwise and counterclockwise rotations over a period of 17 weeks. The deficient animal was given 0.020 or 0.040 cc. of vitamin B(B_1) concentrate each time neuromuscular symptoms (NM) were exhibited.

The frequency distribution of the results of 906 vestibular tests (120 r.p.m.) on 17 rats is shown in figure 4. It is significant that the duration of nystagmus in the normal control animals shows a symmetric frequency curve, while the results on the deficient animals, obtained necessarily at various stages of vitamin deprivation, give an asymmetric or skew curve. Included in the beriberi results are some obtained following treatment with a single dose of vitamin B(B_1) concentrate as noted above. Attention is called to the fact that all the results on the "normal" curve above 18 seconds were obtained upon a single rat which was growing rapidly and may

therefore have had an abnormal vitamin $B(B_1)$ requirement not fully covered by the vitamin $B(B_1)$ in the supplement. Rotation was followed by no nystagmus in 5.3 per cent of the trials on deficient rats, always during recurrent attacks of the acute symptoms. Eye-movements of irregular character and therefore difficult to time accurately comprised 2.3 per cent of the results on deficient animals. No such difficulty was encountered in the normal control group.

Vestibular function tests: second series (60 r.p.m.). In order to eliminate undesirable centrifugal effects occasionally noted in the rotation of larger

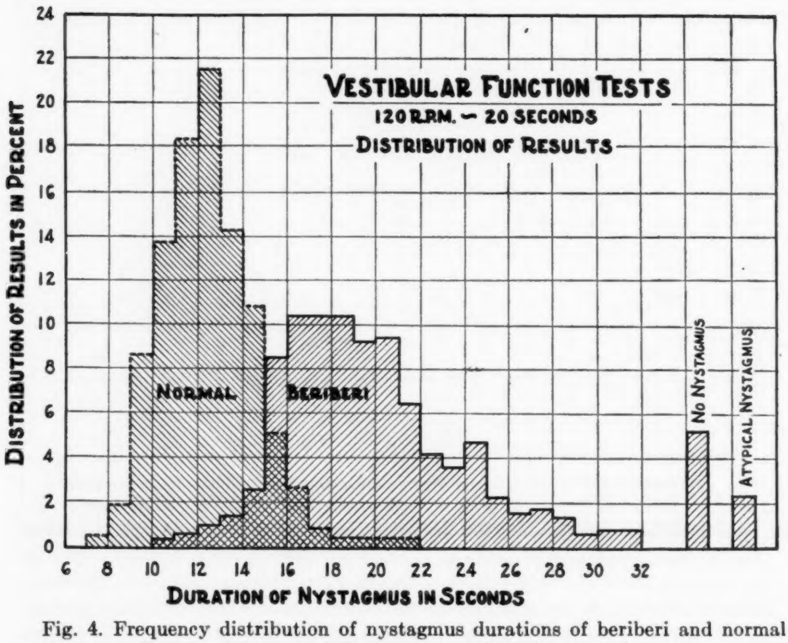

Fig. 4. Frequency distribution of nystagmus durations of beriberi and normal control rats: Results of 906 tests following 4-week depletion period.

rats at 120 revolutions per minute and to save time in making the test, the vestibular function tests of the second series were conducted at a speed of 60 r.p.m. Rotation was continued for 16 seconds, a period which, as shown above, induces the maximum duration of nystagmus at this speed.

As in the first series the animal was rotated in the horizontal plane on a reversible phonograph turntable, with the head held in the normal resting position at the center of rotation. Covering the animal during rotation, so as to eliminate ocular stimuli, was found to be unnecessary. The standard speed was attained, as shown by stroboscopic examination, in about 3

seconds, and the turntable came to a complete but gradual stop within 1 second after applying the brake. The seconds were counted off from the moment of applying the brake to the last pendular movement of the eyes in nystagmus, no attempt being made to estimate fractions of a second. Each test consisted of at least 3 successive observations of nystagmus duration, the direction of rotation being reversed after each observation. The observations in clockwise and counter-clockwise direction were averaged separately and the mean of the two was taken as the result of the test.

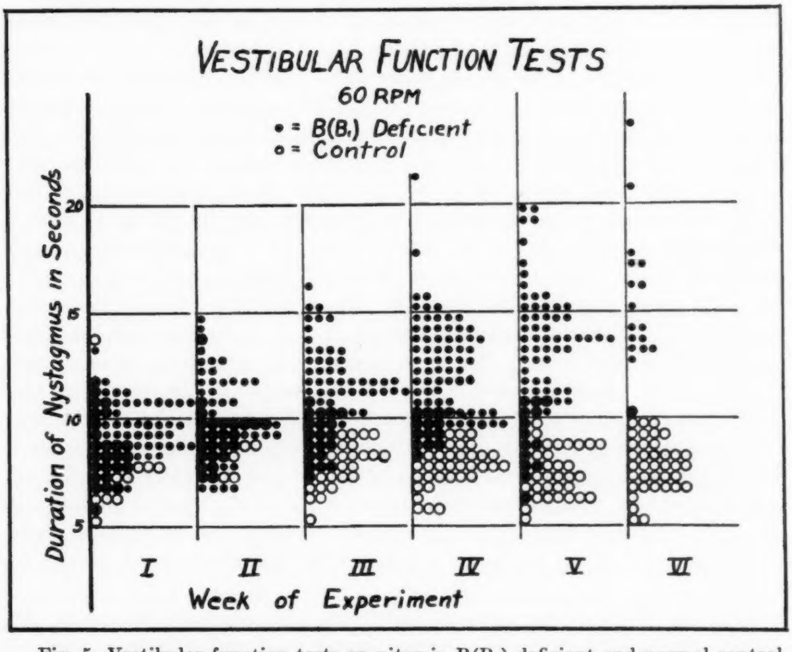

Fig. 5. Vestibular function tests on vitamin-B(B_1)-deficient and normal control rats in successive weeks of the experiment. Spot-chart showing progressive increase in mean nystagmus duration in beriberi prior to the disturbances of equilibrium.

The frequency distribution of the results of 584 vestibular function tests in the second series, showing the duration of nystagmus in 29 animals (18 deficient and 11 controls) in successive weeks of the experiment, is shown in figure 5. The progressive increase in the vestibular response following withdrawal of vitamin B(B_1) from the diet is well established by the third week and reaches the maximum in the fourth to sixth weeks during which period all the deficient animals developed the acute neuromuscular symptoms of beriberi and either died untreated or recovered rapidly after

receiving vitamin B(B_1) in the form of whole yeast. The results in this graph include those obtained up to the stage of disturbed equilibrium but not beyond. The mean duration of nystagmus for each group during the fourth to sixth weeks inclusive was as follows:

Group	*No. of tests*	*Mean duration of nystagmus*
Deficient	161	$12.97 \pm .17$
Control	131	$8.01 \pm .08$

Deficiency in vitamin B(B_1) in this experiment thus resulted in an increase in the mean duration of nystagmus amounting to 62 per cent before the onset of the disturbances of equilibrium.

Effect of inanition upon the vestibular test. The possible effect of partial inanition such as occurs in vitamin B(B_1) deficiency as a result of loss of appetite was investigated by means of a paired-feeding experiment. Six pairs of rats were used, one of each pair receiving diet 31 with cod liver oil and autoclaved yeast, while the other was given an allowance of the same diet equal to that consumed the previous day by its mate, with a supplement of cod liver oil and whole yeast. The weekly results of the vestibular tests, performed on each rat 4 to 5 times a week and averaged for each group, are shown in figure 6. The composite growth curves and the average daily food intake show the degree to which inanition was controlled in the experiment. The growth curves are roughly parallel except for the lag in the downward trend of body weight in the control group, resulting, in part at least, from the difference of one day in the feeding. The progressive increase in the duration of nystagmus preceding the onset of neuromuscular symptoms is again evident. The drop in the vestibular response of both groups during the first 4 weeks is the result of increasing age. It should be noted that the upward trend of the nystagmus curve in the beriberi group begins at the time the animals start to decline in weight.

Relation of otitis to duration of nystagmus. Suppurative disease of the middle ear is of frequent occurrence among rats and it is important to determine its relation to the results of the vestibular tests and to lack of vitamin B(B_1). A unilateral disturbance of labyrinthine function as a result of inner ear disease is occasionally seen in otherwise normal rats, the animals exhibiting asymmetric posture and circling movements. Detlefsen (1923) has shown that rats with "mastoid disease," selected on the basis of their tendency to twist the head and body to one side, gave a slightly longer nystagmus response than the normal when rotated in the direction in which the head was twisted and a much shorter response in the opposite direction.[5] We have obtained similar results with our technic

[5] Those cases showing a right twist of the head gave a much shorter response to counter-clockwise unit stimulus, while those showing a left twist gave a much shorter response to clockwise unit stimulus.

in 4 rats of the breeding colony which showed asymmetric posture as a result of inner ear disease. None of the experimental animals, however, developed any evidence of inner ear disease, although otitis media was frequently found at autopsy. The results of 78 autopsies in which special attention was given to the condition of the middle ears, are shown here:

	Number of autopsies	*Otitis media*	*No otitis media*
Vitamin-B(B_1)-deficient animals	65	33	32
Deficient animals which previously showed unequal nystagmus	11	7	4
Normal control animals	13	5	8
Control animals which previously showed unequal nystagmus	3	3	0

Unequal nystagmus, shorter following rotation in one direction than the other, was the usual accompaniment of otitis media and occurred in both normal and deficient rats. However, the *mean* duration of nystagmus (average of the vestibular responses following clockwise and counterclockwise rotation) was not significantly altered by otitis media, being prolonged in all the deficient animals and normal in all the controls, in spite of the middle ear involvement.

The prolonged nystagmus duration which we have found characteristic of vitamin B(B_1) deficiency is therefore not the result of middle ear disease. Unequal nystagmus, on the other hand, was correlated with otitis media in 71 per cent of the cases.

Qualitative changes in nystagmus. In addition to the increased duration of nystagmus in vitamin B(B_1) deficiency[6], certain qualitative alterations in the vestibulo-ocular reflex have been noted. The eye-movements in the deficient animal are frequently more rapid and the amplitude diminished as compared with the normal rat. Occasionally this was so marked that only with the aid of a lens could the nystagmic movements be detected. Increased rapidity does not accompany increased duration, however; animals with prolonged nystagmus often showed normal nystagmic periodicity. As already noted, the vestibulo-ocular reflex is sometimes absent during neuromuscular symptoms, particularly in recurrent attacks. Treatment with vitamin B(B_1) concentrate was followed in 4 or 5 hours by return of the characteristic eye-movements. In those rats exhibiting spontaneous nystagmus, rotation produced the usual vestibular response, which, if carefully observed, was often seen to be followed immediately by nystagmus in the opposite direction or in a different plane.

The vestibular response in various deficiency diseases. The usual limits of nystagmus duration in the rat at various ages and in several deficiency

[6] Nystagmus duration in vitamin B (B_1), deficient rats just before or during disturbances of equilibrium sometimes reached a value of 20–25 seconds and occasionally as much as 50–150 seconds (60 r.p.m.)

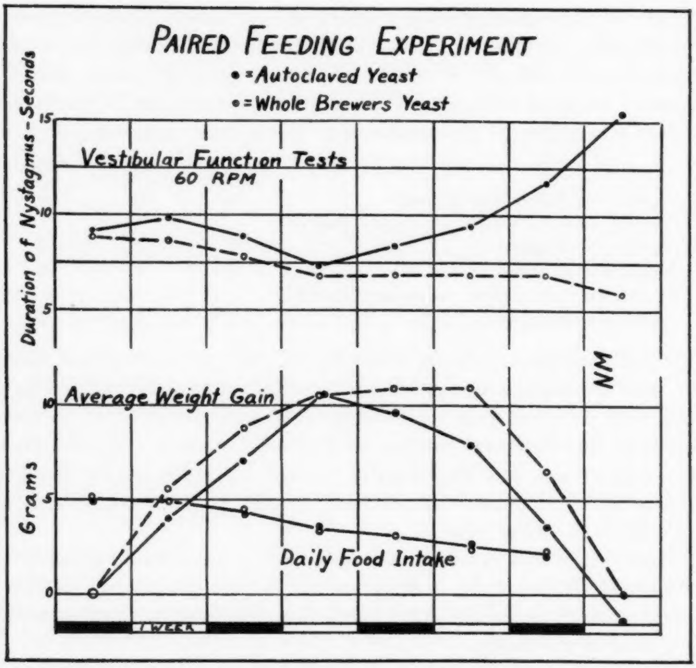

Fig. 6. Paired feeding experiment: Average weekly results of vestibular function tests on six vitamin-B(B_1)-deficient rats and six controls on equivalent food intake. The chart shows the progressive increase in nystagmus duration as a result of vitamin B(B_1) deficiency, and the exclusion of inanition as a factor in this process.

TABLE 2

Nystagmus duration in the rat following rotation at 60 r.p.m.

ON ADEQUATE DIET			
Immature, growing rat	7–10 seconds		
Adult rat	5– 8		
Senile rat	4– 6		
ON DEFICIENT DIET	**INCIPIENT**	**ADVANCED**	**TERMINAL**
Avitaminosis B(B_1)	10–12 seconds	12–16 seconds	Often absent
Avitaminosis A	7–10	5– 7	Occasionally absent
Avitaminosis D	7–10	7–10	7–10 seconds
Avitaminosis G(B_2)	7–10	7–10	9–12*
Fat deficiency	7–10	9–12**	5– 8

* Terminal increase shortly before death.

** Reduced to the normal range by increasing the yeast supplement from 0.54 to 1.0 gram.

diseases are given in table 2. Avitaminosis A results in a slight diminution in nystagmus duration in the advanced stage, and in one animal there was complete loss of the vestibular reflex in the terminal stage. The fat-free diet (31A) supplemented with 2 drops of cod liver oil and 0.54 gram of whole yeast per diem resulted in a significant increase of nystagmus duration. However, when the yeast supplement was increased to 1.0 gram of whole yeast (ether extracted) the vestibular response was reduced to normal. Evans and Lepkovsky (1929) have shown that the vitamin $B(B_1)$ requirement is greater when fat is eliminated from the diet. Our results indicate that the vestibular function test is sensitive to relative insufficiency of vitamin $B(B_1)$ resulting from depriving the animal of fat with its "sparing action" upon vitamin B.

DISCUSSION. A critical examination of the neuromuscular symptoms of vitamin $B(B_1)$ deficiency shows that they consist primarily in loss of proprioceptive function as manifested by alterations in muscle tone, ataxia, and disturbances of equilibrium. True convulsions and paralysis do not, apparently, form a part of the beriberi syndrome. However, the symptoms resulting from vitamin $B(B_1)$ deficiency are often suggestive of paralysis and convulsions and have been often misinterpreted as such. This point is important because the correct interpretation of the symptoms directs the attention to the vestibular apparatus, proprioceptive end-organs, brain stem and cerebellum, with their afferent and efferent tracts, as the possible sites of any nervous lesions produced as a result of vitamin $B(B_1)$ deficiency.

The functional examination of the proprioceptive nerve-endings in the muscles and tendons of normal and beriberi animals gave no indication that these sensory endings or their fibers suffer in consequence of the deficiency. Stretching the muscle and tendon produced afferent impulses in beriberi animals with ataxia and disturbances of equilibrium, as well as in the normal rats, and the oscillograph records were not significantly different. It seems safe to conclude, therefore, that the characteristic symptoms of vitamin $B(B_1)$ deficiency are not the result of functional impairment of the peripheral nerves or nerve endings.

The examination of vestibular function, however, gave results which indicate early and progressive involvement of the vestibular apparatus or of the nerve pathways serving vestibular nystagmus, as a result of withdrawal of vitamin $B(B_1)$ from the diet. The duration of nystagmus following a standardized rotational stimulus increases more than 60 per cent before the appearance of the neurologic symptoms by which beriberi is now recognized. According to Lorente de No (1933) a lesion anywhere in the vestibular system can modify the vestibulo-ocular reflex, which may continue to be produced as long as the reflex arc is closed through one of the multiple pathways. The progressive alteration in vestibular function, terminating in loss of equilibrium and in spontaneous nystagmus or the

loss of one or both components of the vestibulo-ocular reflex, suggests a spreading lesion which involves more and more nerve elements.

Experimental lesions of one or more vestibular nuclei (Fulton, Liddell and Rioch, 1930) give rise to symptoms not unlike those seen in beriberi. These investigators describe marked hypotonia on the side of the lesion with forced rolling movements to that side, asymmetric posture, muscular tremors and spontaneous nystagmus, as sequelae of operative injury to the vestibular nuclei. Flaccidity results, even in decerebrate animals, when Deiter's nucleus is destroyed. Rigidity, on the other hand, is known to result from injury to the red nucleus or the rubro-spinal tracts anterior to the vestibular nuclei. To complete the picture we have only to call attention to the fact that lesions of the cerebellum give rise to ataxia, weakness and muscular tremors. Thus the chief nervous manifestations of beriberi in the rat can be accounted for by lesions within small compass in the brain stem and cerebellum.

Since the publication of our previous report significant evidence has been contributed by Prickett (1934) who found, on histological examination, disseminated foci of hemorrhage or intense congestion of one or both sides involving the nucleus of Deiter's, the chief vestibular nucleus, the nucleus of Bechterew and the nucleus solitarius. While the lesions described lack the specific character to be expected in avitaminosis, the location is consistent with the production of the neurologic symptoms of beriberi. However, the rapid disappearance of the neuromuscular symptoms within a few hours after treatment with vitamin $B(B_1)$ is difficult to reconcile with their causation by hemorrhages and congestion. We have found, on microscopic examination, perivascular extravasations of blood cells in various parts of the brain stem and cerebellum of vitamin-$B(B_1)$-deficient rats killed during the acute neuromuscular symptoms, but we regard these as secondary to the true etiologic process, the nature of which has not yet been established. The work of Peters and his group (1930) on the oxygen-uptake of brain tissue indicates a disturbance of tissue metabolism in vitamin $B(B_1)$ deficiency and offers a possible explanation for the progressive change in vestibular function, as well as for the tissue changes and hemorrhages in the brain stem and cerebellum which occur latter in the deficiency.

Vitamin B_4 deficiency. Reader (1930) described symptoms in the rat resulting from deficiency in a supposedly new factor, vitamin B_4. Animals deprived of the alkali-labile vitamin B fraction (B_1 and B_4) developed polyneuritis in about four weeks. They were then given a dose of B_1 concentrate (Kinnersley and Peters) with disappearance of "convulsions and paralysis" within a few hours. The animal did not gain in weight, however, and remained weak with swollen red paws, spastic gait and loss of coördination. When B_4 (free from B_1) was administered to the rat in

this condition there was immediate gain in weight, an increase in activity, and disappearance of all symptoms within three weeks.

Halliday (1932), following Reader's technic, observed similar symptoms after six weeks on a diet in which protein-free milk supplied the minimum requirements for vitamin B growth factor. The rats walked with a rolling gait and were extremely nervous. They frequently showed red paws and had a tendency to sit in a hunched position. All these symptoms were relieved when whole wheat, or an acid extract thereof, was fed. The curative factor has been isolated in crystalline form, which in physical and chemical characteristics is closely similar to Reader's vitamin B_4, although not as yet identified with it (Halliday, 1934).

Doctor Halliday very kindly sent us two of these rats showing symptoms and also permitted us to examine animals in her laboratory. These animals exhibited various stages of neuromuscular disturbance, including marked alterations in muscle tone, ataxia, disturbances of equilibrium, hyperexcitability, pathologic tail reflex, and greatly prolonged vestibular response (17 to 27 seconds at 60 r.p.m.). It appears, therefore, that the effects on nerve function of deficiency in Halliday's factor differ in no essential respect from those resulting from lack of the heat-labile vitamin B of yeast. Two alternative explanations may be offered for this apparent identity of effects: Either 1, the deficiency syndrome produced by Halliday and probably also that by Reader is the result of partial or incomplete deficiency in vitamin $B(B_1)$, or 2, the neurologic disturbances resulting from lack of the heat-labile vitamin B of yeast are the consequence of deficiency, not of the growth-promoting, appetite-stimulating vitamin $B(B_1)$, but of a second factor present in the heat-labile fraction of yeast, possibly vitamin B_4. In the absence of evidence clearly proving the latter hypothesis, our tentative conclusion is that the neuromuscular syndrome produced by Halliday and by Reader is the result of partial deficiency in vitamin $B(B_1)$.

SUMMARY AND CONCLUSIONS

1. The characteristic neurologic symptoms of beriberi resulting from lack of vitamin $B(B_1)$ in the rat are 1, changes in muscular tonus; 2, ataxia; 3, disturbances of equilibrium, and 4, hyperexcitability. Muscular tremors and weakness sometimes occur but true convulsions and paralysis are not part of the beriberi syndrome.

2. The labyrinthine righting reflex is lost in beriberi at the onset of disturbances of equilibrium but the neck and body righting reflexes are preserved. A pathologic tail reflex exhibited by beriberi rats gives evidence of the spreading of the reflex arc in the spinal cord.

3. Evidence is presented of the functional integrity of the peripheral nerves and proprioceptive nerve endings in beriberi in the rat by the method of recording nerve action potentials.

4. Vestibular function is significantly altered in beriberi. The duration of nystagmus following a standardized rotational stimulus increases progressively following withdrawal of vitamin $B(B_1)$ from the diet, preceding the appearance of other neurologic symptoms.

5. This prolongation of vestibular response is characteristic of vitamin $B(B_1)$ deficiency. Inanition, otitis, and deficiency in vitamin D do not appreciably affect the mean result of the vestibular test, while deficiency in vitamin A diminishes the result. In vitamin $G(B_2)$ deficiency a small terminal increase was noted.

6. The vestibular function test is sensitive to the relative insufficiency of vitamin $B(B_1)$ resulting from depriving the animal of fat (with its "sparing action" upon vitamin B).

7. The chief neurologic manifestations of beriberi in the rat can be accounted for on the basis of lesions in the vestibular nuclei. The finding of perivascular hemorrhages in this region is confirmed but these lesions are regarded as secondary to insidious tissue changes resulting from specific lack of vitamin $B(B_1)$.

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THE INFLUENCE OF ELECTROLYTES ON RESPIRATION IN NERVE

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The many influences of various ions, alone and in combination, upon the colloidal structure and physiological attributes of nerve and other tissues have been exhaustively studied (see Gerard, 1932; Graham, 1933). Little has as yet been done, however, in exploring such influences on the metabolic activity of nerve. Such information would be of considerable significance in two respects. On the one hand, the pictures of nerve activity which are current coinage make rather definite statements about changes in structure and metabolism during conduction; statements which can be further checked by data upon these points. Clearly, the manner in which respiration changes in response to experimental conditions must ultimately be equated with changes in other properties to the same conditions.

Secondly, and more immediately interesting, is the question of the tie-up between metabolic and other events in nerve. The problem has been considered elsewhere: what factors fix the actual respiratory rate for a tissue, and which lead to sudden changes in it, as during activity (Gerard, 1932, 1934). The answer involves such items as the structural and chemical organization in which the oxidations proceed—colloidal dispersion, membrane permeability, ionic strength and composition, and the like.

For any integrated understanding of the chemical and physical events associated with the wave of activity in a nerve fiber, such data as we have obtained are a necessary preliminary. Part of this material has appeared in brief reports (Gerard, 1930; Chang, Gerard and Shaffer, 1932a, b); and the results show that the common tissue cations and anions do play significant rôles in regulating respiration, somewhat similar to those found for irritability, and that the mechanism of their action is, on the whole, via effects on the colloidal structure of the nerve.

METHODS. The Warburg manometric method was used throughout. Ordinarily ten manometers, eight with attached chambers of about 5 cc. volume, two of 10 cc., were used at a time. All were provided with a central cup, in which 0.1 to 0.2 cc. N/10 NaOH was placed to absorb CO_2. Some were further provided with side-arms, from which special reagents

could be tipped. In the main body of each chamber were placed about 100 mgm. of nerve in enough solution to give with the tissue 1.0 cc. (doubled in larger vessels), except for one chamber, serving as pressure and temperature control, from which tissue was omitted. The manometers were filled with air in experiments with frog nerve, with oxygen for dog nerve. The effect of a special reagent was obtained by comparing the respiration before and after tipping the reagent from the side-arm into the main chamber, or by comparing the respiration of matched nerves (from the same animals) with and without the reagent added to the main solution. In a few cases results from different individual frogs were compared. Oxygen consumption is expressed throughout as a rate, (Qo_2), cubic millimeters of oxygen consumed per hour per gram of fresh tissue.

Using pure isotonic saline (or Ringer) as the basic solution, all others are expressed as isotonic percentage of each salt added. Thus "10 per cent KCl" means 10 parts isotonic KCl to 90 parts isotonic NaCl (or Ringer). Solutions were freshly prepared by mixing stock solutions, and were brought to the desired pH (usually 7.4) with Sorenson's phosphate buffer (1:10) or by direct titration. Final pH was checked with the quinhydrone electrode.

Nerves were rapidly dissected after pithing and bleeding the green frogs, two being ordinarily used per chamber. They were placed so that two chambers each contained one nerve of the pair from each frog. Most of the experiments on frog sciatics were performed at 21°C., a few at 25°C., and some at 37°C.; all those on dog vagus at 37–38°C. It is important to note, in evaluating respiration results, that nerves from the same frog may differ by 10 per cent, from different ones by 100 per cent, under the same conditions. Of course, consistent differences of even less than 10 per cent become significant. All statements of fact in this report are based on several consistent (at least three) duplicate experiments, though in some cases only one is given in the tables. In all, about one thousand runs were made.

Results. A. *The effect of alkali-salts.* 1. *NaCl.* It has long been known that the frog sartorius will twitch when soaked in isotonic NaCl solution. Riggs (1919) claimed that frog nerves produced an extra amount of CO_2 under such treatment, but his actual data were not convincing. Gerard (1930), using the Warburg technique, did not find a corresponding increase in oxygen consumption. As NaCl is present in sea water and all biological fluids to over 80 per cent of the total salt concentration, it was important to make this point certain. The results of several series of experiments warrant the conclusion that at 20 to 25°C. isotonic NaCl has no definite effect on nerve respiration, not over a 5 per cent increase at most and irrespective of whether or not the nerve sheath is split to insure rapid penetration. At 37–38°C., however, the effect is very marked (see table 1).

At 37° both frog and dog nerve show an increase in oxygen consumption in NaCl as compared to Ringer. (No phosphate buffer was added to the Ringer in these experiments, since at the desired pH much of the Ca^{++} in the solution, 0.024 per cent $CaCl_2$, or tissue would be precipitated by the anions. Ringer with phosphate added acts about the same as pure saline, the effects of which, therefore, are largely related to calcium lack.) The values in saline are regularly high from the start (by $\frac{1}{4}$ to $\frac{1}{3}$), but become strikingly higher after four or five hours, when the tissue exhibits a great increase in oxygen consumption. In Ringer this increase is less and occurs two or three hours later than in saline. This late increase in respiration is not due, as we first believed, to a form of ionic and osmotic injury to the tissue, exaggerated by the low calcium and consequent greater permeability of cell membranes, but to bacterial growth. This question is discussed more fully in the following paper, but it should be pointed out here that the earlier interpretation may be in part correct. The increase in oxygen consumption due to bacterial growth is definitely modified, in time and quantity, by the presence or absence of calcium ion. This is no longer true when the nerve is killed by boiling at the start of the run—in this case the bacterial rise is much accelerated and calcium has no influence. The calcium, then, appears to determine the speed with which the nerve disintegrates, gives off nutrient substances or permits bacteria to invade it, rather than directly affecting bacterial growth. The action is, thus, in the usual direction—the cell integrity being maintained longest in balanced salts, diffusion increasing in calcium lack, and a complete disintegration resulting from thermal death. The influence of sodium, per se, will be brought out in connection with the use of non-electrolyte solutions.

TABLE 1

NERVE	TEMPERATURE	TIME	Q_{O_2}	
			Ringer	NaCl
		hours		
Frog, intact (2 exp.)	21°	20	40	43
Frog, split (3 exp.)	25°	7	53	55
Frog, intact (3 exp.)	38°	4	94	96
		Next 5	44	182
Dog vagus (3 exp.)	37°	5	143	308
		Next 4	243	372

2. *Potassium salts.* Riggs (1919), Gerard (1930) and Schmitt (1931) have reported that the respiration of frog sciatic nerve is depressed about 50 per cent by isotonic KCl. Gerard also used 10 per cent isotonic KCl in saline, and found no effect. The present results are in accord, and we

have further determined the potassium effect over the whole range of concentration. Average results, shown in the accompanying graph (fig. 1) indicate a practically maximal effect at 50 per cent KCl. After being kept in Ringer in the ice box for 18 hours, following a period in potassium mixture, all the nerves showed good action potentials. Also, respiration again increases in Ringer, so the potassium depression is clearly reversible. The concentration required to produce a marked effect on respiration is higher than that which is required to markedly decrease irritability. The latter, according to Misske (1930), is from 5 to 7 per cent, while a 10 per cent solution is not sufficient to cause a detectable depression in oxygen consumption.

A similar effect of K^+ was also found with dog vagus, an average depression of 68 per cent being obtained in isotonic KCl. For muscle (frog sartorius) the case is very different. In confirmation of Fenn (1931), we have found that an excess of potassium increases respiration. Pure isotonic KCl solution leads to increases of about two-thirds above the value in Ringer (Q_{O_2} of fifty experiments in Ringer = 38), and even the addition of 30 per cent of this solution causes a distinct increase.

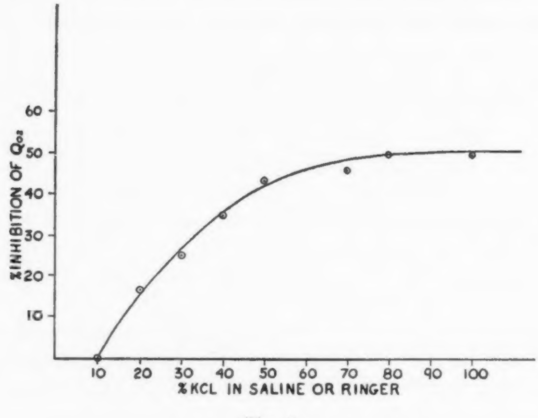

Fig. 1

3. *Rhubidium and caesium.* Both these ions depress nerve respiration, though not so much as potassium. In 50 per cent isotonic solution (with sodium) there is little effect; in pure isotonic solution of their chlorides, each gives a 25 per cent inhibition.

The respiration of muscle is likewise depressed by these ions, especially caesium, despite a vigorous twitching. This is in contrast to the increase associated with potassium contracture.

B. *The effect of alkaline earths.* 1. *Calcium.* The maximal depressant

effect of this electrolyte on the Q_{O_2} of the frog's sciatic, as previously reported (Gerard 1930), is about the same as that of KCl (50 per cent.) Its effect on the dog's vagus is somewhat less (32 per cent). When graded concentrations are used, the maximal depression is obtained with 80 to 100 per cent isotonic $CaCl_2$. In many experiments, no change resulted from 50 per cent solutions and only a 30 per cent depression appeared with

TABLE 2

SOLUTION	ISOTONIC	INCREASE IN Q_{O_2}	NUMBER OF EXPTS.	SOLUBILITY (Ca^{++}) IN H_2O AT 25°C., MGM. PER CENT Ca^{++}
	per cent	*per cent*		
NaOxalate				0.2
	20	74	2	
	30	90	2	
	100	88	2	
NaCitrate				0.8*
	0.5–2	9	2	
	5	19	1	
	10	54	5	
	20	52	2	
	35	107	3	
	50	82	3	
	100	76	11	
NaFluoride				0.9
	30	25	3	
NaTartrate				8.0
	10	0	1	
	20	9	1	
	35	24	2	
NaPhosphate				10
(buffer, pH 7.8)	10	15	15	
	100	40	50	
NaSulphate	100	7	8	53

* Estimated from data of McLean and Hastings, 1933.

80 per cent solutions; in others even 50 per cent solutions gave nearly maximal depressions. Misske (1930) obtained a marked decrease in irritability with 25 per cent $CaCl_2$ in saline but not with 10 per cent. Hence the effect on respiration is again less than that on irritability. The respiration of brain slices (dog and rabbit, 38°) is also depressed by excess calcium.

1a. *The effect of decalcifying salts.* It is clear that excess of free Ca^{++}

in the medium tends to lower respiration, as well as irritability, and results in pure NaCl suggest the converse. The Ca content of nerve is higher than that of the blood or lymph which bathe it -0.13 to 1.2 per cent of the fresh weight of rabbit sciatic (Kraus, etc., 1924); 0.6 to 1.5 per cent of frog (Simon and Szelöczey, 1928; Fenn, 1934, gives the more probable figure 0.025—still well over 0.01, for serum). Decalcifying salts, by rapidly reducing the Ca^{++} concentration inside the axones themselves as well as in the outside medium, serve to extend the results obtained by simply omitting calcium from the medium. The effect varies with the nature and amount of active anion added. All such anions lead to an immediate increase of respiration even at 21°C. and, especially interesting, the increase tends to vary inversely as the free Ca^{++} concentration that remains in each case.

TABLE 3

SOLUTION	Q_{O_2} (8 HOURS)	NUMBER OF EXPTS.
$\frac{CaCl_2}{NaCl}\ \frac{8}{1}$	33	(3)
$\frac{CaCl_2}{NaCitrate}\ \frac{8}{1}$	32	(3)
NaCl	50	(1)
$\frac{NaOxalate}{NaCl}\ \frac{1}{1}$	71	(2)
$\frac{NaOxalate}{NaCitrate}\ \frac{1}{1}$	77	(2)

TABLE 4

SOLUTION	Q_{O_2} FOR 6 HOURS	DECREASE
		per cent
Ringer	33.0	
$\frac{CaCl_2}{KCl}\ \frac{(2)}{(8)}$	18.7	43
Ringer	34.6	
$\frac{CaCl_2}{KCl}\ \frac{(5)}{(5)}$	13.4	61

With Na citrate (23 experiments and controls) below 30 per cent isotonic in Ringer or saline, the effect is present but not definitely maximal. Even 1 per cent citrate, however, gives a 10 per cent increase. At 30 per cent or above, a maximal increase is obtained, from 70 to over 100 per cent. The results with various anions are briefly summarized in table 2.

Since anions in general tend to increase respiration, permeability, etc., the effect increasing with valence (Mathews, 1904; Lucké and McCutcheon, 1932), the citrate ion might act in these experiments by its peptizing effect on negative or its coagulating effect on positive colloids, aside from any relation to calcium. The following tests, however, demonstrate that such an effect, if present, was very small. In one series, citrate was added, or not, to an excess of calcium. Its presence was immaterial, so at least it is not effective as a complex (table 3). Another point, 10 to 20 per cent isotonic citrate produces as marked an effect as any higher concentration. This would combine with all the calcium ion (0.004 M)

and should therefore be a maximal concentration on this basis; whereas stronger solutions might be expected to produce greater effects if dependent on colloidal changes (see table 2). Finally, when respiration is increased by addition of oxalate, the further addition of citrate has little effect (table 3). This would be expected if calcium removal is the critical point, not if anion charge is, for, of course, the citrate ion is trivalent, oxalate only bivalent.

As already noted (table 2), oxalate alone gives about the same increase in respiration (60–90 per cent) as does citrate alone. The respiration increase in Na tartrate (about 25 per cent) or Na fluoride (25 per cent), moreover, is definitely less than that in oxalate or citrate, in harmony with the higher free Ca^{++} they permit, though phosphate gives an intermediate effect. (A particular, depressive action of fluoride may also enter into the action of this ion. See, e.g., Dickens and Simer, 1929.) Though Riggs (1919) has emphasized the stimulating effect of sodium sulphate, one would predict in terms of decalcification that, because of the solubility of $CaSO_4$, sulphate could not be very effective in augmenting nerve respiration. In the present experiments with isotonic sodium sulphate at 20°C., the respiration of frog's sciatic nerve increased under 10 per cent in 7 to 9 hours (table 2), as compared to NaCl controls; nor did splitting the nerve sheath alter the results. During a further 10 to 12 hours the results were irregular, fluctuating about normal by 10 to 30 per cent. Sulphate, therefore, exerts at best a small positive effect, in harmony with the relatively great solubility of its Ca salt.

1b. *Calcium and potassium.* Ringer with the calcium removed behaves like isotonic NaCl, so that the small potassium concentration of the former appears to be without influence on the calcium action. In view of the marked antagonism between Ca^{++} and K^+ on many properties of nerve, it seemed desirable to explore rather fully their combined effects on respiration. Direct combination of both ions, in excess concentration, does not lessen the independent depressive action of either one alone (table 4).

Menten (1912) reported that potassium salts whose anions remove calcium have no depressive effect when injected into various mammals, which would speak for synergy rather than antagonism. Experiments performed on isolated nerve with potassium salts of these anions largely agree (table 5). The depressant action of K^+ in potassium citrate and KH_2PO_4 was as marked as in KCl, but recovery on placing the nerves in Ringer was better after these than after the chloride. When, further, KCl and sodium citrate were mixed in varying ratios, the depressant effect was much lower in mixtures rich in citrate than in pure KCl, even allowing for dilution of the K^+ (table 6). These experiments demonstrate an antagonism between potassium and citrate, probably primarily via calcium ion changes. But since 10 per cent citrate should suffice to give a maximal decalcifying

effect, the further increase of citrate action with increasing concentration suggests, also, a direct antagonism between the cation potassium and the anion citrate.

1c. *Calcium on dog vagus.* The dog vagus even more than the frog sciatic, at 38°, behaves quite differently in normal Ringer and in decalcified Ringer. In the latter, the Q_{O_2} is considerably higher from the start and at the 3rd to 5th hour a further rise is nearly always seen. A rise in ordinary Ringer also often appears, but it is small and much delayed. In serum, the initial respiratory rate is high and no late rise occurs (see the following paper), but addition of sufficient Ca^{++} remover causes a further increase in oxygen consumption (table 7).

Muscle respiration is similarly increased by addition of decalcifying salts, up to 200 per cent; and the increase evoked by potassium is also

TABLE 5

SOLUTION	Q_{O_2} (FOR 4 HOURS)	Q_{O_2} (FOR 3 HOURS) AFTER PLACING IN RINGER	INCREASE AFTER PLACING IN RINGER
			per cent
KCl	13.2	18.8	42
K-citrate	16.2	28.4	75
KCl	23.3	26.7	15
K_2HPO_4	22.0	37.2	69

TABLE 6

SOLUTION	Q_{O_2} IN 10 HOURS	DEPRESSION IN KCl OR KCl-CITRATE MIXTURE
NaCl	39.2	
$\frac{\text{KCl (60)}}{\text{NaCitrate (40)}}$	33.8	14
NaCl	33.4	
$\frac{\text{KCl (80)}}{\text{NaCitrate (20)}}$	24.3	27
KCl	16.2	55
$\frac{\text{KCl (90)}}{\text{NaCitrate (10)}}$	25.7	29

augmented by these anions, up to twice that due to potassium alone. Calcium and potassium together give smaller increases than potassium alone, and excess calcium leads, often after an initial increase, to a marked falling off in respiration.

2. *Magnesium.* $MgCl_2$, used in amount equivalent to the $CaCl_2$, has practically the same effect on nerve respiration and seems fully able to replace this ion. In excess of magnesium, respiration is depressed 45 per cent. It is noteworthy that this equivalence does not extend to the grey matter of the brain, magnesium ion appearing indifferent to the respiration of this tissue despite its well known "anesthetic" action. In eleven experiments on dog or rabbit brain, the Q_{O_2} for the first hour or two averaged 950. This fell in the fourth hour by 27 per cent with no addition, by 28 per cent after addition of magnesium (to 0.03 M), and by 48 per cent

after addition of a similar amount of calcium salt. (It is interesting that the central nervous system is especially sensitive to low calcium. Flushing the dorsal cavity with NaCl, let alone the use of Ca removers, gives convulsions in cats. (Weed and Wegeforth, 1916.)

3. *Barium.* The stimulating action of this salt on muscle has long been known, and powerful twitchings have been obtained with as little as M/30,000 $BaCl_2$ in saline (Chao, 1934). On nerve respiration its action, like the other alkaline earths, is depressive, giving in 35 per cent isotonic solution (0.03 M) a decrease of 30 to 50 per cent, which progresses in

TABLE 7

Dog vagus and sciatic, 37°C.

SOLUTION	NUMBER OF EXPTS.	DURATION	Q_{O_2}	CHANGE
		hours		*per cent*
$\frac{\text{Serum}}{\text{NaCl}}\ \frac{9}{1}$	3	8	189	
$\frac{\text{Serum}}{\text{Na-KPO}_4}\ \frac{9}{1}$	3	8	192	
Serum	3	12	156	
$\frac{\text{Serum}}{\text{NaCitrate}}\ \frac{9}{1}$	3	12	177	+13
$\frac{\text{Serum}}{\text{NaCl}}\ \frac{7}{3}$	3	4	157	
$\frac{\text{Serum}}{\text{NaCitrate}}\ \frac{7}{3}$	3	4	224	+45
$\frac{\text{Serum}}{\text{NaCl}}\ \frac{7}{3}$	3	8	212	
$\frac{\text{Serum}}{\text{NaOxalate}}\ \frac{7}{3}$	3	8	275	+30

TABLE 8

SOLUTION	Q_{O_2} (6 HOURS)	DECREASE
		per cent
NaCl	36.0	
M/50 $AlCl_3$ in NaCl	14.2	60
NaCl	41.0	
M/100 $AlCl_3$ in NaCl	16.7	59
NaCl	35.0	
M/200 $AlCl_3$ in NaCl	13.3	62

later hours to 60 or 75 per cent. The effect of $BaCl_2$ on grey matter is similar: concentrations from 0.5 to 10 M give a decrease of about 10 per cent.

C. *The effect of a trivalent cation* ($AlCl_3$). The powerful coagulating effect of aluminium salts (probably related to the precipitating action on negative colloids) was at once apparent from the appearance of nerves immersed in dilute $AlCl_3$. The depressant action on respiration was marked at much lower concentrations than in the case of the alkaline-earths. A maximal decrease (60 per cent) was obtained at concentrations

as low as M/200, and probably this was still considerably above that required for a maximal effect (table 8). A somewhat smaller decrease (40 per cent) was obtained in two experiments on dog's vagus.

It is worth noting that citrate which, presumably, disperses colloids, increases the weight of nerves immersed in it, while aluminium, with the reverse effect, decreases nerve weight.

D. *The effects of monovalent anions.* Mathews (1904) maintained that the effect of any given salt depends on the balance of opposed effects of the contained cation and anion, a view that has received strong support in the experiments of Lucké and McCutcheon on the cobaltamines (1932). Both ions are surely concerned in producing effects on structural colloids. For nerve, Netter (1927) found the axiolemma impermeable to anions, which also have little effect on the resting potential. Höber and Strohe (1929) found on frog's sciatics that an electrotonic current decreased slowly after a brief rise in NaCl, rapidly in CNS^- or I^-; NO_3^- and Br^- acted like Cl^-; $SO_4^=$ had no effect; and the addition of 0.02 per cent $CaCl_2$ to any of these rendered its effect negligible. They also found that the presence of $CaCl_2$ largely cancels the action of the same salt series on nerve irritability. Under such conditions, I^-, Br^-, NO_3^- and $SO_4^=$ caused a very slight increase followed by a negligible drop, CNS^- an immediate fall or no change for some time.

In harmony with the above, we have found the respiration of nerve largely indifferent to these anions. This is true for the frog's nerve (at 21°C.) as well as the dog's (at 38°C.). NaSCN and especially $NaNO_3$ have, however, a distinct effect (table 9).

On muscle, these anions have greater effects, leading to increases up to 400 per cent. The order of diminishing effectiveness is: CNS, I, SO_4, Br, NO_3, Cl.

E. *The effect of isotonic non-electrolyte* (*cane sugar*). In association with the loss of irritability of muscle in sugar solution, Fenn (1931) found a marked increase in respiration. Nerve loses its irritability more slowly in sugar solution, though splitting the sheath hastens this considerably (Feng and Gerard 1930), and respiration is depressed rather than increased. Also, the respiration changes much sooner in intact nerve than does the irritability. A depression of 10 to 45 per cent (average 26 per cent) is usually present within one to two hours, and is then maintained or increased for over twenty hours (21–25°C., frog's sciatic). Splitting the sheath has no effect on the speed of action on respiration. Dog vagus shows a similar but smaller depression, 22 per cent in two experiments. After exposure to the sugar solution, nerves become translucent and rather stiff.

E1. *Sugar and salt mixtures.* For muscles, the loss of irritability and the increased respiration in sugar solution are reversible phenomena, being

restored to the original level when the tissue is transferred into saline or Ringer (Overton, 1904; Fenn, 1931). The loss of irritability of nerve in sugar solution is also reversible, and hence is presumably due to the diffusion out of electrolytes.

In accord with expectations, addition of NaCl to the sugar largely prevents the fall in respiration. Addition of KCl or $CaCl_2$, on the contrary, does not restore the Q_{O_2}, but may further depress it. Ten to 30 per cent by volume of NaCl in sugar solution has but little effect on the initial sugar

TABLE 9

Influence of anions (lytropic series)

Frog sciatic (21°C.)

SOLUTION	Q_{O_2}	DURATION	INCREASE
		hours	*per cent*
NaCl	39.5	5	
NaBr	39.6	5	—
NaCl	46.6	7	
NaI	48.8	7	—
NaCl	40.6	8	
$NaNO_3$	50.0	8	23
NaCl	42.0	8	
NaCNS	44.7	8	+

Dog vagus (37.5°C.)

	Q_{O_2} FOR 4 HOURS	INCREASE
		per cent
Ringer	223	
NaBr	225	
NaI	227	
$NaNO_3$	246	10
NaCNS	271	21

depression, but when a 40 to 60 per cent solution is used, this depressant effect is largely removed. Later, even 20 per cent NaCl is effective in preventing the further fall in pure sugar; and the respiration in 40 per cent NaCl is usually 20 to 30 per cent higher than in pure saline. The presence of ions (NaCl) is clearly essential to the respiration of nerve. NaCl likewise restores in an hour the normal opacity to a nerve rendered translucent in sugar. The stiffness, however, persists after 13 hours.

In sharp contrast to the findings on muscle, addition of KCl to sugar fails to restore the normal respiration, but their depressive effects sum

(table 11). The same was found for calcium in a single experiment (1 calcium to 3 sugar cut the Q_{O_2} 30 per cent) and depressions are progressive with time.

Extending previous observations, in five experiments with nerves in NaCl and glucose, addition of insulin (10^{-5} to 10^{-1} units) had no effect on respiration. Glucose alone also has no influence in moderate concentration.

F. *Weight changes.* Isolated tissues are not in equilibrium with isotonic saline solutions, and it is interesting to note the influence of various

TABLE 10

Frog sciatic (25°C.)

SOLUTION	EXPTS. NO.	DURATION	Q_{O_2}	CHANGE
		hours		*per cent*
Ringer	3	7	51.6	
M/4 sucrose	3		40.9	−21
NaCl*	3	20	31.5	
M/4 sucrose*	3		18.0	−43
NaCl	15		42	
M/4 sucrose	11		31	−26

* Frogs long in captivity.

TABLE 11

Frog sciatic, 20°C. 7 to 20 hours. Q_{O_2} in Sucrose = 28

Solution	Sucrose / NaCl	10/0	9/1	8/2	7/3	6/4	0/10
Q_{O_2}		100%	100+%	125%	140%	150%	160%
Solution	Sucrose / KCl	10/0	7/3	3/7			
Q_{O_2}		100%	80%	60%			

ions on the swelling of nerves, though no systematic study has been made. Frog nerve, at 20°, gains about 8 per cent in weight during 1 to 3 hours in frog Ringer. Even in mammalian Ringer there is a slight gain, about 5 per cent, despite its hypertonicity. In dog serum, frog nerves maintain their initial weight for days. Pure isotonic potassium chloride leads to enormous swelling, up to doubling in thirty hours, which is largely reversed by subsequent treatment with NaCl or Ringer. Weight increases of 20 per cent follow immersion in NaCl for 10 or more hours. In isotonic calcium solutions there is little weight change for 10 hours, followed by swelling to about 20 per cent increase in another twelve hours (fig. 2).

Pure lithium or caesium acts like Ringer, giving but small weight increases. Swelling in sucrose is about the same as in NaCl, as it is also in the following sodium salts: iodide, thiocyanate, tartrate and citrate. Bromide and nitrate and possibly sulphate give less swelling than the chloride. Further data on dog nerves in saline and protein solutions are presented and all discussed in the following paper.

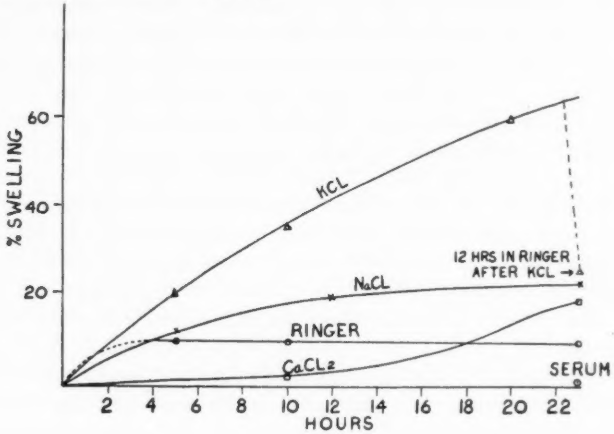

Fig. 2

Discussion. In most experiments supporting the existence of a stimulating effect of sodium chloride, pure isotonic solution has been used. Apparently the out-diffusion of Ca^{++} from the plasma membrane or the interior of the cell has not always been considered. But Ca-lack is far more effective in causing dispersion of structural colloids and in increasing permeability than is Na-excess. At lower temperatures this process seems to be exceedingly slow in nerves, each fiber being wrapped in a myelin sheath and neurilemma. This may explain the absence of any measurable effect on nerve respiration in isotonic NaCl at 21°C. in a period of twenty hours. At higher temperatures (37–38°C.) the changes are greatly accelerated, so that in saline the respiration of the nerve is higher at the start and rises further, as a rule in 3 to 5 hours, than when in Ringer.

The important rôle of NaCl in nerve is shown by the effects of its absence or deficiency rather than by its excess. Thus, in sugar solution, as soon as the major amount of this salt is removed from the interstitial fluid or the plasma membrane, the oxygen consumption is decreased. The respiration decrease occurs long before irritability loss, to which it may contribute. A certain amount of the NaCl in the medium is necessary to maintain the normal respiration as well as irritability of nerve.

The effect of KCl on nerve respiration is atypical, for its action on structural colloids, like that of Ca-lack (Höber and Strohe, 1929), is to increase dispersion and so the permeability of the tissues. Also potassium excess acts, like cathodal polarization (Woronzow, 1925; Mackuth, 1926; Graham, 1933), to give a temporary increase followed by a decrease of resting potential, and electrotonic current spread. Irritability, though ultimately depressed, is for some time definitely raised—especially to long current pulses (Blumenfeldt, 1925; Höber and Strohe, 1929). But respiration is uniformly and permanently depressed, no temporary rise having appeared. (A brief effect at the start would, however, be missed.) Similarly, potassium somewhat diminishes the spike action potential and strongly the after-potential; prolongs absolutely and relatively refractory periods, and slows conduction (Graham, 1933). Graham gives no indication of early changes in the opposed direction, though occasionally a long after-potential accompanies potassium treatment. KCl is effective on nerve respiration only in excess; its absence apparently has no effect. Any complete removal, however, as of calcium by citrate, was not obtained. The oxygen consumption of skeletal muscle, on the contrary, is greatly increased by KCl (Fenn, 1931; Hegnauer, 1933). This is most probably due to the contracture induced by this salt, and the fundamental effect may be a depression. This is at least the case for heart muscle, the respiration (and tone) of which is decreased by excess potassium (Arnoldi, 1924; Victor, 1933), as in nerve.

$CaCl_2$ exerts an effect in both excess and deficiency. Its absence or decrease leads to a rise in oxygen consumption; its excess to a fall. These changes parallel reasonably those of irritability, etc., as well as the clinical manifestations associated with hypo- or hypercalcemia. Here again the respiration of heart is affected as is nerve, that of skeletal muscle inversely.

The more prompt and intense change in respiration obtained on addition of decalcifying agents, as compared with simple absence of Ca from the medium, may be interpreted as the difference between active and passive removal. As the decalcifying ions penetrate, Ca^{++} is locally reduced to very low concentrations, permeability is heightened, and further penetration follows. A rapid invasion of the axone and prompt loss of Ca^{++} results.

On the whole, the effects of ions on nerve respiration can be interpreted in terms of their effects on the colloidal structure of the nerve. The cations lessen colloidal dispersion, decrease enzyme surface activity, and depress respiration. The anions evoke opposite colloidal and respiration changes. In addition, specific ion effects, as for calcium, often play a primary rôle.

Of all nerve properties so far studied, the only ones changing from the start in the same direction when acted on by excess of either potassium or

calcium are conduction velocity, respiration and, possibly, spike height. There is obviously little ground, on the basis of the available data on salt action, for drawing extensive conclusions as to the interrelations of the phenomena attendant on nerve function. The tedious exploration of the action of sodium, calcium and potassium ions, in varying amounts and ratios on most nerve properties still awaits adequate performance. There is, however, no question that the amount of these ions and their distribution between the inside and outside of cells play a predominant rôle in controlling nerve conduction, sense organ discharge, and reflex activity. (Gerard, 1932; Feng, 1933; Talaat, 1933; Huggins and Hastings, 1933; Boyd and Ets, 1934; Cowan, 1934; etc.)

SUMMARY AND CONCLUSIONS

1. The influence of several common ions on nerve respiration has been investigated. In general their action on respiration is closely related to their effect on the colloidal structure of nerve.

2. Sodium is important in maintaining the normal oxygen consumption of nerve. In its absence respiration is decreased 10 to 40 per cent, a stimulating effect with excess is doubtful.

3. Potassium manifests a depressant action only in excess (maximal depression 50 per cent at 50 per cent solution). Its absence has no effect. Such a depressant effect on respiration is not parallel to irritability and some other changes in nerve induced by potassium.

4. Calcium is especially important since small increases or decreases in its concentration have a marked influence on nerve respiration and irritability. Respiration, like irritability, varies inversely with calcium content.

5. Magnesium, but not barium, is able to substitute for calcium in nerve; neither can in grey matter.

6. All the decalcifying sodium salts increase nerve respiration, the increase roughly paralleling decalcifying power, from 25 per cent with tartrate and fluoride to about 100 per cent with oxalate and citrate.

7. Aluminium, the only trivalent cation studied, depresses nerve respiration and has a strong coagulating effect.

8. Most monovalent anions are of little or no influence, possibly related to their inability to penetrate the nerve membrane.

9. Isotonic sugar solution depresses nerve respiration to 50 per cent. Addition of NaCl restores it to normal, while addition of KCl or $CaCl_2$ further depresses, thus giving additive effects with the sugar.

10. No antagonism on nerve respiration is manifest between sodium and calcium or potassium and calcium, but cations and anions do antagonize.

11. The results favor the common view that a structural colloid of nerve is negatively charged.

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THE INFLUENCE OF BLOOD CONSTITUENTS ON OXYGEN CONSUMPTION IN NERVE[1]

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In the preceding paper (Chang, Shaffer and Gerard, 1935) the actions of a number of ions on nerve respiration have been examined. The present work is directed to a study of other blood constituents normally present and able to act upon nerve in vivo. We were led to this study in part by the observation that the rate of oxygen consumption of nerve, and other tissues, was at first distinctly higher when suspended in serum than when in Ringer, but that after some hours a marked rise in the latter medium reversed the order. In part, also, other observers had noted differences in the behavior of isolated tissues in serum and in artificial media, and a further study of the factors involved gave promise of interest. Although the work was largely carried out on nerve, other tissues were found to behave often more strikingly, and the findings are of interest for vertebrate tissue and cell respiration in general.

We had early observed an increased respiration of nerve in saline or in buffered Ringer over that in unbuffered Ringer, with a marked further rise at the end of three or four hours. These changes were related to Ca^{++} deficiency, since addition of Ca^{++} diminished the higher respiration in saline and delayed, while not entirely preventing, the later rise. Since calcium might be lost by combination with nerve phosphates, serum was also used, as a medium with more normal free and bound calcium concentrations. Serum completely prevented the later rise, but, although it contained an adequate amount of Ca^{++}, led to a definitely higher initial respiratory level than did Ringer (see fig. 1, for dog vagus). The present study showed the later rise of nerve respiration to be due to bacterial growth[2] (prevented by moderately heat-labile bactericidins, Pettersson,

[1] Preliminary reports of this work were published in Science **76**: 571, 1932; and Science, Supplement on nerve, 1934.

[2] Bacteria could be seen under the microscope in large numbers, after 5 hours at 37°. The final proof that the rise was due to bacteria was obtained by performing several experiments with full aseptic precautions, when it did not occur. Sterile nerves in Ringer continued to consume oxygen at a very slowly diminishing rate for a week, when the experiment was stopped. Clearly the "fuel" was not carbohydrate for this period (see Gerard, 1932), as 3 per cent of the moist nerve weight would be required.

1927–8, in the protein fraction of the serum), though the *time* of this rise may well be related to the action of Ca^{++} in maintaining the structural integrity of the nerve. The initially high values in serum are found to be related in large part to the presence of proteins.

Since this work was begun, others have made similar observations on brain (Quastel and Wheatley, 1932; Karczog, 1933) and nerve (Rosenbaum, 1932). Quastel and Wheatley attribute the difference between Ringer and serum to the presence in serum of glucose and lactate, both of which do increase the respiration of brain. Rosenbaum (1932) regards an increased respiration of nerve in blood over that in Ringer as due to the presence of erythrocytes. That these are minor factors, if they are factors at all, in nerve will be indicated by the results to be presented. Karczog (1933) has found the respiration of brain to be nine times greater in serum ultrafiltrate than in Ringer. This also is contrary to our findings for nerve, though other tissues do behave somewhat similarly.

Serum as a medium more closely approaches the conditions in vivo than does Ringer, and it may at first appear incorrect to speak of an increase in serum rather than a decrease in Ringer. However, the greater part of the studies on irritability as well as on respiration are based on the values in Ringer as standard, and it seemed wise to retain the convention. Further, besides the difficulty of obtaining adequate amounts of frog serum for the work with frog tissues, the use of Ringer (or similar solutions) offers the advantages of a simple medium of known and constant composition. Nevertheless, it has perhaps been too readily assumed that the respiration in saline media is quite comparable with that in vivo, and other components should receive more attention in future work.

Method. The modified Warburg method (Gerard, 1931) has been used throughout. In each series six to fourteen manometers were used, most with chambers of about 5 cc. volume (a few double this), and with a central cup in which was placed 0.1 cc. of M/5 NaOH for the absorption of CO_2. From 40 to 60 mgm. of tissue were used in each chamber, and enough solution added to make a final volume (including the alkali) of 1 cc. of liquid and tissue (2 cc. in the larger vessels). Tissue was omitted from one chamber which served as the temperature and pressure control, in addition to the semi-differential arrangement of the manometers. Except in a few experiments with frog nerve, the chambers were filled with oxygen, since the oxygen tension of air is too low for thick mammalian nerves. Unless other temperatures are indicated, all values given are for 37.0 to 38.0°C., the variation being between separate experiments, not in the course of one. Also, unless specified, Q_{O_2} values are for the vagus nerve of the dog and represent the average rate for the first three to five hours of the run, or as long as a fairly uniform rate was maintained.

Since, as mentioned, the addition of 10 per cent isotonic phosphate

buffer increases respiration, unbuffered Ringer solution was used throughout. Any variations in pH are not likely to be of importance, however, for we have found no observable difference in the respiration of dog nerve between pH 6.8 and 7.6. For frog nerves, pH values between 6.0 and 9.0 are indifferent (Gerard, 1930). As the pH of serum is usually above 7.4 after standing for a short time, the Ringer was adjusted to pH 7.56 by direct titration, checked with the quinhydrone electrode.

Frogs were pithed and eviscerated prior to tissue removal. Dogs were lightly etherized and then bled from one of the carotid arteries. The

TABLE 1

Q_{O_2} in mammalian Ringer and serum, 37°C.

TISSUE	AVERAGE Q_{O_2} IN RINGER	AVERAGE Q_{O_2} IN SERUM	T	PER CENT INCREASE IN SERUM
			°C.	
Dog:				
Vagus nerve	172 (53)	234 (49)	38	36
Sciatic nerve	138 (4)	207 (2)	38	50
Phrenic nerve	123 (2)	168 (2)	38	36
Sympathetic chain	143 (1)	230 (1)	38	60
Striated muscle (diaphragm)	263 (5)	353 (5)	38	34
Liver	298 (5)	645 (5)	38	116
Kidney	1,498 (5)	1,680 (5)	38	12
Testicle	368 (2)	515 (1)	38	40
Choroid	179 (1)	467 (1)	38	160
Retina	710 (1)	2,040 (1)	38	187
Liver	230 (1)	520 (1)	27	126
Frog:				
Sciatic nerve	112 (6)	133 (6)	38	19
Sciatic nerve	125* (2)	202 (2)	38	69
Smooth muscle (stomach)	93 (1)	148 (1)	27	59
Liver	214 (3)	378 (3)	27	76
Kidney	425 (1)	800 (1)	27	87

* Frog Ringer.

blood was defibrinated by gentle stirring and centrifuged to obtain the serum, or was centrifuged at once and serum obtained above the clot in the centrifuge tube. The nerves were rapidly dissected and kept in a beaker of serum, usually warmed. Each tissue was cut into sufficiently thin or short pieces, which were surface-dried with filter paper and weighed on a torsion balance. Manometers containing control and experimental solutions were paired to contain adjacent pieces of the same nerve, and, further, the pair was reversed in repeating a series. Thus any constant errors in the apparatus and random or regular variations in the material were cancelled out in an adequate series.

Results. Average results for a number of different tissues in Ringer and in serum are given in table 1. Numbers in parenthesis indicate the number of experiments on which the Q_{O_2} value is based. Only experiments with Q_{O_2} values in Ringer and serum for the same nerve are included.

No statistical significance can be attached to individual values other than those for the vagus nerve and for liver (five pairs of experiments for livers, tested by the Chi square method, have a probability of 0.02), but the invariable rise of respiration in serum encountered in a wide variety of tissues is fully convincing. Further points are based on a number of paired runs giving uniform and consistent results.

The complete data obtained on vagus nerve are too extensive to be repeated here, but table 2 contains typical values. Each figure is for a single experiment and each horizontal pair from the same nerve, and so directly comparable. The figures underlined are the extreme minimal and maximal values obtained.

TABLE 2

Q_{O_2} for vagus nerve in Ringer and serum

RINGER	SERUM
164	233
186	212
190	243
167	209
185	218
185	227
203	249
204	288
125	277
254	288
101	158
154	328

The 36 per cent difference between the averages in Ringer and in serum (table 1) is not due to a difference in the range of variability. There is comparatively little overlap in the figures; thus: only 9 of the 53 values for Ringer are above 200 and only 4 of the 49 values for serum are below 200; and in the experiments which yielded these more extreme Q_{O_2}s the relative differences between Ringer and serum were still maintained. These variations are to be attributed to individual differences between dogs as to age, physical condition, and perhaps sex. (Younger animals, for example, show a relatively larger respiration.)

The difference between two pieces of the same tissue in the same solution is usually less than 10 per cent, though occasionally as much as 30.

Table 3 is a protocol of one typical series, illustrating the degree of constancy and variation obtained.

In attempting to identify one or more active factors in such a complex as serum, either its constituents may be removed one by one, testing the remaining fluid, or they may be added singly to Ringer solution and so tested. The former was first undertaken.

The removal of lipoids from serum was effected by four extractions with ether, followed by aeration (with a mixture of 95 per cent oxygen and 5 per cent CO_2) to remove ether traces. Two series of experiments indicated that this treatment had no effect: first series, Q_{O_2} = 176 (2) in whole serum and 181 (5) in ether-extracted serum; second, Q_{O_2} = 204 (3) in fresh whole serum, 202 (4) in preserved (2 days) whole serum, and 207 (5) in preserved (2 days) ether-extracted serum. Any remaining trace of ether would have decreased respiration, as was further checked in a control experiment: Q_{O_2} = 256 (2) in serum, 179 (2) in Ringer, and 120 (2) in Ringer with a trace of ether. Although the method used does not effect complete removal of serum lipoids (and may also remove as much as 6 per cent of the total calcium; Louchs and Scott, 1929), it seems unlikely that the lipoids are an important factor in producing the higher Q_{O_2} in serum.

TABLE 3

Q_{O_2} in Ringer and in serum for dog liver and kidney

MANOMETER NUMBER	TISSUE	MEDIUM	Q_{O_2} AVERAGE FOR 4 HOURS
1	Kidney	Ringer	1,473
2	Kidney	Ringer	1,657
3	Kidney	Ringer	1,543
4	Kidney	Serum	1,820
5	Kidney	Serum	1,968
6	Kidney	Serum	1,821
7	Liver	Ringer	249
8	Liver	Ringer	263
9	Liver	Serum	690
10	Liver	Serum	716

Removal of proteins was first attempted by heating the serum (in a water-bath) to 85°C. and centrifuging the coagulated proteins from the clear fluid. The first results indicated no effect: Q_{O_2} = 226 (3) in whole serum and 235 (7) in protein-free serum. But since it is reported (Csabo and Faubl, 1924) that heat precipitation of proteins removes a large part of the calcium with the coagulum, a rise in Q_{O_2} due to calcium lack might mask a fall due to protein loss. (See preceding paper.) When $CaCl_2$ was added to the heated serum, a distinct fall appeared: Q_{O_2} = 241 (1)

in fresh serum and 179 (2) in heated serum. This procedure has objectionable features, however, aside from the disturbance of the salt content, as heating to 85° will destroy most of the enzymes and may have other effects. An attempt was made to check the enzyme factor by heating to 60° for forty minutes, sufficient to destroy them without coagulating the proteins. The Q_{O_2} in serum was not significantly affected by this treatment: Q_{O_2} = 184 (2) in fresh serum and 172 (4) in heated serum.

Removal of the proteins by ultra-filtration was accomplished by the use of porcelain filters covered with a collodion (7 per cent) membrane with

TABLE 4

Q_{O_2} of dog vagus in serum ultra-filtrate

MEDIUM	AVERAGE QO_2 FOR 7 HOURS
Ringer	186
Ringer	166
Serum	212
Serum	198
Serum	204
Ultra-filtrate	184
Ultra-filtrate	144
Ultra-filtrate	158

TABLE 5

Q_{O_2} of dog vagus in serum ultra-filtrate

MEDIUM	AVERAGE QO_2 FOR 4 HOURS
Ringer	190
Ringer	202
Ringer	157
Serum	243
Serum	239
Serum	209
Ultra-filtrate	167
Ultra-filtrate	192
Ultra-filtrate	173

TABLE 6

Q_{O_2} of dog vagus in serum ultra-filtrate

	SERUM	SERUM ULTRA-FILTRATE	RINGER
1	235 (7)	180 (8)	160 (4)
2	230 (3)	177 (3)	183 (3)
3	205 (2)	162 (3)	176 (2)
4	182 (1)	122 (1)	112 (2)
Weighted average	225 (13)	172 (15)	160 (11)

pores of sufficient size to insure the diffusibility of everything in serum except the proteins, the non-diffusible calcium (probably bound to the proteins in non-ionized form—Greenburg and Gunther, 1928; McLean, 1934), and possibly some of the magnesium (Greenburg and Gunther) and lipoids. (We are indebted to Dr. Julian Lewis for aid in preparing these filters.) The ultra-filtrates gave negative tests with Millon's reagent and are thus to be considered protein-free. Tables 4 and 5 are protocols of two series of experiments, showing that the ultra-filtrate behaves like Ringer.

In table 4 the serum used as a control and from which the filtrate was prepared had been preserved at 4°C. for four days. A large number of experiments have shown that the property (or properties) of serum responsible for the higher respiratory level is not impaired by such preservation.

Averages from four series of experiments (including the above data) are given in table 6. No consistent difference between the Q_{O_2} in Ringer and in the ultrafiltrate is observable, while the average for serum is 31

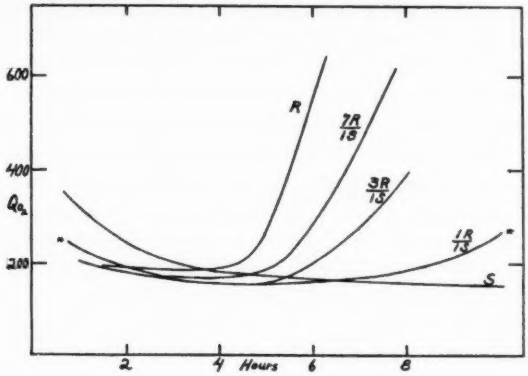

Fig. 1

per cent higher than the ultrafiltrate. (The probability, calculated for pairs by the Chi square method, is 0.013.)[3]

[3] Frog or dog liver at 27° shows almost as high a Q_{O_2} in heated serum as in whole serum. E.g., frog liver Q_{O_2}: Ringer = 189 (4); serum = 375 (2); coagulated serum = 325 (5). Also, in sharp contrast to nerve, cerebrospinal fluid, fresh or boiled, maintains respiration at the same level as whole serum—Q_{O_2} = 372 (6). Hemoglobin added to Ringer likewise has far more effect on liver or kidney respiration than on that of nerve. (See also Solandt, Solandt and Gerard, 1934, on mytilus gill respiration.)

	RINGER	HEMOGLOBIN RINGER	SERUM
Dog liver, 37°	230 (1)	370 (3)	520 (3)
Frog liver, 37°	485 (1)	535 (1)	755 (1)
Frog liver, 27°	260 (1)	385 (1)	710 (1)
Frog kidney, 37°	690 (1)	795 (1)	1,160 (1)
Frog kidney, 27°	425 (1)	570 (1)	800 (1)
Dog nerve, 37°	175 (7)	181 (11)	221 (2)

For other tissues than nerve, therefore, different factors play the determining rôle. Particularly, the diffusible heat stable substances of serum are more important.

The positive result of removing proteins was suggestive. The alternate method of testing individual serum constituents by addition to Ringer solution was next followed.

The addition of 0.1 per cent glucose to Ringer does not increase the Q_{O_2} of dog or frog nerves. (See Gerard, 1932; Sherif and Holmes, 1930.) In one series, a mixture of equal parts of Ringer and serum (= 0.05 per cent glucose) gave a Q_{O_2} 26 per cent higher than did Ringer with 0.1 per cent glucose added.

The addition of glycine to Ringer and to serum does increase the Q_{O_2}. As the increase in Q_{O_2} only roughly follows the glycine concentration, average figures for several concentrations (0.01 to 1.0 per cent) are given: Q_{O_2} = 198 (2) in Ringer, 238 (4) in Ringer with glycine, 232 (3) in serum, 268 (4) in serum with glycine. The increase in Ringer is 20 per cent and that in serum, 15. Kisch (1931, 1932, 1933) reports similar results with glycine, glycyl-glycine, alanine, phenylalanine, lactic acid, etc., for retina and other tissues. It seems unlikely, however, that the amino acids are an important factor in the respiratory increase of nerve, since their concentration in serum is only 0.004 per cent, and further, being readily diffusible, they must have been present in the serum ultra-filtrate.

Four other serum constituents which produced no appreciable effect on addition to Ringer were urea (25–50 mgm. per cent), sodium lactate (100 mgm. per cent), sodium glycocholate (5 mgm. per cent), and creatin (20 mgm. per cent).

The addition of lipoids to Ringer was not very satisfactory, due to solubility difficulties. A 0.17 per cent suspension of crude lecithin produced, however, as much as 75 per cent inhibition of respiration. This may depend on the presence of free choline as an impurity, since choline, or acetyl choline, does inhibit: Q_{O_2} = 160 in Ringer and 110 (6) in Ringer with 0.01 to 1.0 per cent acetyl choline; Q_{O_2} for rabbit sciatics = 234 in Ringer with 0.001 per cent acetyl choline and 97 in Ringer with 0.01 per cent acetyl choline. "Choline-free" lecithin, however, also produced a 20 per cent depression: Q_{O_2} = 287 (2) in serum, 240 (3) in Ringer and 195 (4) in Ringer with 17 per cent choline free lecithin. The marked inhibitory effect of acetyl choline is interesting, since it is closely related to an immediate end product of phospholipin or myelin breakdown and may be concerned in the active metabolism of nerve as well as in many cases of neurohumeral transmission.

The addition of serum proteins to Ringer should afford the critical evidence necessary for an affirmation of the ultra-filtration results, but the method used in obtaining the proteins was not adequate. They were precipitated by acidifying serum to the isoelectric point (pH ca. 5.4), separated by centrifuging, washed several times with Ringer and, in Ringer suspension, readjusted to a pH of about 7.2 On standing, a large part of

the protein (apparently undenatured) went back into solution, but the amount thus dissolved never approached the concentration in serum, even though large amounts of serum were used in the preparation. (This is borne out by observations on weight changes in the nerves, which will be considered later.) The results (table 7) failed to show any influence of serum proteins on the respiratory level.

Experiments with more concentrated protein solutions, however, give quite different results. Three series on the *addition of egg-white to Ringer* are summarized in table 8. The egg-white, from fresh eggs, was well stirred with Ringer to a homogeneous solution. Nerves in this solution respired about as in serum. A control on egg-white alone (no nerve)

TABLE 7

Effect of serum proteins in Ringer

TISSUE (DOG)	Q_{O_2} IN RINGER	Q_{O_2} IN RINGER + PROTEIN	Q_{O_2} IN SERUM
Vagus nerve	220 (3)	239 (3)	256 (3)
Vagus nerve	226 (3)	215 (3)	234 (3)
Vagus nerve	256 (4)	230 (3)	
Diaphragm	213 (2)	264 (3)	402 (2)
Diaphragm	253 (2)	237 (3)	339 (2)
Diaphragm	233 (4)	239 (3)	

TABLE 8

Q_{O_2} in Ringer with egg-white

RINGER	RINGER AND EGG-WHITE	EGG-WHITE	SERUM
168 (2)	220 (2)		262 (1)
179 (4)	258 (1)	211 (3)	207 (3)
113 (2)	199 (2)		191 (2)

showed no oxygen consumption, whereas nerves in pure egg-white respired as in serum. Results pointing in the same direction were obtained by the addition of commercial blood albumin to Ringer. Thus, $Q_{O_2} = 187$ (2) in Ringer, 214 (2) in serum, 176 (1) in serum ultra-filtrate, and 205 (3) in serum ultra-filtrate + 6 per cent albumin. Gelatin likewise led to small but consistent increases. The gelatin solution was quite viscous and poorly stirred and oxygenated, which would account for the relatively small increase.

Since it can hardly be assumed that such proteins as egg-white and gelatin serve a nutrient rôle in nerve oxidations, the manner in which proteins act to increase respiration was sought among physical factors. The weight change of nerves was followed in many experiments. The greater

the respiration, in general, the less weight increase. Nerves in Ringer ordinarily gained 19 to 40 per cent of their original weight during an experiment (3–7 hours)—the gain not being proportional to time. Nerves in serum gained nothing to 25 per cent of their original weight; in Ringer with 6 per cent gelatin, under 2 per cent. Glycine, which also increases oxygen consumption (although here actual oxidation of the glycine may be involved, in addition to any physical effects), exerted a definite osmotic effect. On the other hand, the Ringer solutions of acid-precipitated proteins (which gave no increase in oxygen consumption) led to increases in weight from 19 to 50 per cent, in the same range as Ringer alone. It thus

TABLE 9

Q_{O_2} in Ringer with 6 per cent gelatin

TISSUE	RINGER	RINGER + 6 PER CENT GELATIN	PER CENT INCREASE
Vagus nerve	259 (4)	283 (4)	9
Sciatic nerve	228 (4)	253 (4)	11

TABLE 10

Increase in weight of nerve in Ringer or serum + glycine

MEDIUM	PER CENT INCREASE IN WEIGHT IN 8 HOURS
Ringer	39.0
Ringer + 0.01% glycine	33.0
Ringer + 0.1% glycine	30.0
Ringer + 1.0% glycine	23.0
Serum	14.0
Serum + 0.1% glycine	14.5
Serum + 1.0% glycine	10.0

appears that a medium that increases the respiration decreases the swelling of a tissue.

A number of experiments designed to determine the time relations of this increase in weight were carried out on the dog vagus and rabbit sciatic and brachial. Nerves transferred directly from the animal to Ringer gained about 10 per cent of their original weight during the first ten minutes, and 20 per cent or more by the end of the first hour, after which further increase was small or absent. In serum, the increase was only 1 to 2 per cent during the first ten minutes and 8 to 11 per cent by the end of an hour. The rapidity with which these changes take place suggests that the absorption of water may be at first largely into the intercellular tissue spaces. The reason for the absorption of water from serum is not

clear—unless some breakdown of cell constituents occurs (following injury due to removal from the animal and to cutting) which increases intracellular osmotic pressure. Acid liberation would have a similar effect. (Shafer, 1933, finds a 15 per cent swelling of muscle fibres on fatigue.) In any case, the effective osmotic pressure of the medium must be due largely to the serum colloids, as the crystalloids can (though slowly) pass through the nerve sheath and plasma membrane; and even in strongly hypertonic (2 times isotonic) Ringer nerves eventually gain 19 per cent of their original weight.

Experiments with Ringer solutions of varying osmotic pressure indicate that hypertonicity (up to 2 times isotonic) has little effect on oxygen consumption. Hypotonicity does not produce a definite effect until the osmotic pressure is reduced to one-half isotonic. Rosenbaum (1932) also reports no effect of osmotic pressure on mammalian nerves within a range

TABLE 11

Q_{O_2} in Ringer + serum

TISSUE	SERUM	RINGER	½ RINGER + ½ SERUM	¾ RINGER + ¼ SERUM
Dog vagus	363	204	241	200 195
Dog vagus		162 165		216 197
Dog liver		248 355		550 496 516

of ½ to 1½ times isotonic, and Gerard (1930) found that the Q_{O_2} of frog sciatics is not changed in Ringer up to four times isotonic, though hypotonic solutions depress increasingly, to a respiration one-third normal in distilled water. Thus, merely increasing the osmotic pressure by the addition of crystalloids will not increase the respiration. The higher oxygen consumption in serum, then, if due to physical factors alone, is related to the colloid osmotic pressure of the serum proteins, which presumably serve, as salts can not, to oppose osmotic forces drawing water into the tissue. This recalls Adolph's (1931) interesting observation that the frog's gastrocnemius swells in pure NaCl below 1.0 per cent concentration. With 6 per cent gelatine added, however, equilibrium is established with 0.65 per cent NaCl, though the protein osmotic pressure is negligible. Even dilute serum is effective; table 11.

Discussion. The higher respiratory rate in serum is independent of

the activity or activability of nerve, since it is maintained long after conduction is abolished; and similar effects are obtained with other inactive tissues. There is clearly an influence on the cell metabolism as such. Other investigators have suggested blood constituents, other than protein, as the basis of the increased respiration in serum or blood. Although glucose and amino-acids may well be factors in raising the respiration of brain, liver and other organs, this cannot well be so for medullated nerve. These substances must have been present in the serum ultra-filtrates which showed a nerve respiration similar to that in Ringer, nor has direct addition of them to Ringer increased respiration. Rosenbaum's observation that erythrocytes increase respiration can offer no explanation for the results with serum; and a repetition of his experiments, using washed erythrocytes in Ringer did not show an increased respiration. As reported, however, hemoglobin may augment the respiration of tissues. A possible rôle of serum enzymes, hormones and the like has not been eliminated by the ultra-filtration experiments, as these might not pass the filter. However, serum heated to a temperature that will destroy most enzymes supports a respiratory rate similar to that in fresh serum; adding proteins to Ringer gives an increased oxygen consumption in the absence of any enzymes; and the addition to serum of an easily oxidized substrate like sodium acetate (in the absence of tissue) does not result in oxygen consumption.

Though the evidence from the addition of proteins to Ringer is not alone conclusive, the further evidence from ultra-filtration experiments strongly suggests that the serum proteins are important factors in producing the increased respiration in serum. The mechanism by which they produce this effect is more uncertain. Being too large to penetrate the nerve sheath or plasma membrane, they can hardly play a nutrient rôle.

Surprisingly little is known about the rôle of the serum proteins in the intact organism. It has been demonstrated that the maintenance of a minimal plasma protein concentration is essential to life (Whipple, Smith and Belt, 1920); but this probably depends on the need for a protein osmotic pressure to balance hydraulic pressure and so regulate fluid balance between capillary blood and tissue lymph. It is dangerous to draw a parallel between this and the situation in vitro, where the tissue is directly bathed by a colloidal solution of the blood proteins but no blood pressure exists. However, these workers offered evidence that "the essential injury in these experiments is cell protoplasm injury induced by a sudden change in the colloidal solution which forms the normal environment of these cells." They also observed that certain cells, especially liver, were particularly sensitive to changes in serum protein content, as has also appeared in these respiration studies. But a piece of frog stomach muscle is irritable after three hours in mammalian Ringer; and also the marked

difference between tissue (e.g., table 1) suggests that the higher respiration in serum is related to a more specific effect on cell metabolism than is implied by "cell protoplasm injury." Kerr (1929) reports that a dilution of the serum proteins greatly increases the permeability of red blood corpuscles to sodium and potassium. And, while other factors are not excluded, the rôle of the serum proteins may be to preserve the normal state of the semipermeable membrane and so, by preventing a rapid exchange of ions between the cell and medium, the normal state of the cell protoplasm.

It is interesting to recall the serious tissue injury resulting from the perfusion of intact organs and tissues with artificial salt solutions, and the old observation (Guthrie, 1908) that organ transplantation is unsuccessful if the transplanted organ is washed out with normal saline before the blood flow is reëstablished. Recently, however, Amberson and others (1933) have maintained heart beat, respiration and other activities for seven hours in a completely perfused animal (dog or cat) by adding hemolyzed blood to the Ringer-Locke perfusing solution, the hemoglobin serving to maintain colloid osmotic pressure as well as to carry oxygen.

SUMMARY

1. The oxygen consumption of dog nerve is, on the average, 36 per cent greater in serum than in Ringer solution. Other tissues show a similar, but quantitatively specific, behavior; increases varying up to 116 per cent for liver and even more for retina.

2. An analysis of the serum constituents indicates that this increased respiration is related to the serum proteins, in the case of nerve. (The increase in liver, etc., is not related to proteins.) Glycine in Ringer (in sufficient concentration) leads to an increase; lecithin and acetyl-choline to marked decreases. Urea, creatin, lactic acid and glucose are indifferent to nerve respiration.

3. The proteins probably play a physical rôle via osmotic effects and maintenance of the normal semi-permeability of the plasma membrane. This is suggested in part by the ability of serum to prevent weight increases which occur in "isotonic" salt solutions.

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THE MECHANISM OF THE ARSENITE ACTION ON MEDULLATED NERVE

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Although it has been long known that arsenite paralyzes motor and sensory nerves and spinal reflexes (Ringer and Murrel, 1878; Lesser, 1878; Lendle and Reinhardt, 1931) the mechanism of the action is unknown. Since the action is irreversible it is usually supposed to be necrobiotic and as such of little interest. Schmitt, Skow and Bueker (1934) studied the effect of arsenite on the electric properties and on the respiration of frog nerves and concluded that the effects could be explained best by assuming a poisoning action on oxidative or other enzyme systems of nerve. Warburg (1925) assumed that arsenite inhibits respiration by combining with heavy metal atoms of the respiratory enzyme. Szent-Györgyi (1930), obtaining no inhibition of indophenol oxidase by arsenite, rejected this explanation and came to the conclusion (Banga, Schneider, and Szent-Györgyi, 1931; Banga and Szent-Györgyi, 1932a, b) that the inhibition is due to blocking of substrate activation. Voegtlin believes that arsenite interferes with oxidative processes by forming a stable union with sulphydryl compounds in tissues (Voegtlin, Dyer and Leonard, 1923; Rosenthal and Voegtlin, 1930; Voegtlin, Rosenthal and Johnson, 1931). In the present paper we have confined ourselves to a study of the action of arsenite on the oxygen and substrate activating systems and on the sulphydryl complex.

1. Oxygen activation. Holmes (1930) found that while the oxygen consumption of washed nervous tissue was extremely low, addition of p-phenylenediamine produced marked increases due to the presence of oxidase. The oxidase activity of peripheral nerve, however, was only a few per cent of that of brain and hence it is not surprising that we have found the oxidase activity of cold blooded peripheral nerve too low for measurement in this manner. We used instead a modification of the colorimetric method of Dye (1927), based on the oxidation of the Nadi reagent. Two or three nerves are placed in a 25 cc. flask and 10 cc. of freshly prepared Nadi reagent added, the flask attached to a shaking rack placed in the thermostat and shaken for ten to thirty minutes. The nerves are then withdrawn from the flask, washed for a brief period in water to remove adhering dye,

and transferred to a 10 cc. volumetric flask containing 5 cc. 70 per cent alcohol (to arrest the activity of the enzyme and to extract the color from the nerves). This flask is then transferred to the thermostat and shaken until the dye has been extracted from the nerves (about 30 minutes). The volume is made up to 10 cc. with 70 per cent alcohol and read colorimetrically using indophenol blue in 70 per cent alcohol as a standard. A control must be run on boiled nerves to correct for the dye which becomes absorbed, since the reagent undergoes a small amount of autoxidation and this tends to produce some coloration even in boiled or cyanided nerves. The results of these experiments show that arsenite in millimolar solutions not only fails to inhibit the oxidase but may actually accelerate it by as much as 25 per cent above the rate of the control nerves. Although the indophenol oxidase test cannot be used rigorously as a measure of the activity of the respiratory enzyme, nevertheless, in view of the relatively high degree of inhibition of respiration produced by arsenite in intact nerve, these results indicate that the effect is on some system other than the respiratory enzyme. This is confirmed by the fact that methylene blue, which may be considered equivalent to active oxygen and which antagonizes cyanide inhibition, has little effect on arsenite inhibition of nerve respiration.

2. Substrate activation. Thunberg (1923) and Sherif (1930) demonstrated the presence of dehydrogenases in mammalian nerves by the methylene blue technique and the latter author found that narcotics prolong the decolorization time from 50 to 150 per cent. We are unaware of any experiments on substrate activation in cold blooded nerves. In our experiments 100 to 300 mgm. of unwashed nerve, cut into bits or crushed, were placed in each Thunberg tube, to which was added 1 cc. of water (or arsenite), 0.2 cc. phosphate buffer, and 0.1 cc. methylene blue, the tube evacuated and placed in the thermostat in the dark[1] and the decolorization time noted. It will be seen from table 1 that, like narcotics, arsenite inhibits substrate activation considerably. For maximum prolongation of the decolorization time it is necessary to soak the nerves in arsenite for several hours before evacuation. This preliminary incubation may be related to a similar procedure described by Banga, Schneider and Szent-Györgyi (1931) who found that arsenite prolongs the decolorization time of methylene blue only if the tissue has been given a preliminary period of incubation. Nerve differs, however, from heart muscle, which was used by them, not only in respect to the intensity of oxidation but also to the substrates used in resting metabolism and hence this similarity may not mean a similarity of underlying mechanism. In nerves this incubation period appears to

[1] It is essential that these experiments be done in the dark for we have found that nerve will decolorize methylene blue under the influence of light whether boiled or unboiled and with or without evacuation. These effects are similar to those described by Whitehead (1930) for milk and unsaturated fatty acids.

be more closely associated with the induction period previously noted in connection with the extinction of the action potential by arsenite (Schmitt, Skow and Bueker, 1934). Arsenite inhibition differs from that of typical narcotics such as urethane which produce their inhibition without great time lag. Arsenite is also considerably more potent than urethane on the nerve system. While M/100 arsenite may prolong the decolorization time several hundred per cent, M/10 urethane under similar conditions is practically without effect; concentrations of the order of M/2 urethane are required. The effect of arsenite on substrate activation in frog spinal cord was found to be similar to that on peripheral nerve.

TABLE 1

The effect of arsenite on methylene blue reduction time of nerve

DATE	WEIGHT OF NERVE		CONCENTRATION OF METHYLENE BLUE	CONCENTRATION OF ARSENITE	TIME IN ARSENITE	TEMPERATURE	REDUCTION TIME		INCREASE IN REDUCTION TIME
	Control	As					Control	As	
	mgm.	*mgm.*		*M.*	*min.*	*°C.*			*per cent*
8/24/33	269	262	1:65,000	0.00077	5	26	55	58	6
8/23/33	256	257	1:65,000	0.00077	24	26	61	70	15
8/21/33	255	245	1:65,000	0.00077	53	26	87	87	0
8/22/33	267	266	1:65,000	0.00077	93	26	62	132	113
9/ 5/33	110		1:40,000				211		
		110	1:40,000	0.000125	132	25		237	12
		110	1:40,000	0.000625	132	25		256	21
		110	1:40,000	0.00125	132	25		291	38
1/25/34	111	111	1:90,000	0.01	269	35	36	74	105
	115	115	1:90,000	0.01	269	35	36	211	487*
1/26/34	123	125	1,90,000	0.01	300	35	39	82	110
	123	126	1:90,000	0.01	300	35	46	298	548*

* Nerves taken from winter frogs which had been kept at 25°C. for 23 days prior to the experiment.

With winter frogs the time necessary for arsenite block of the action potential may be greatly shortened by warming the frogs for several days previous to the experiment. As shown in table 2, the extinction time may be shortened as much as 65 per cent in this manner. And, while the methylene blue reduction time of nerves from winter frogs previously warmed may be somewhat longer than that of nerves from cold animals, treatment with arsenite greatly increases the difference. Thus in one typical experiment, the reduction times were as follows: winter nerves untreated = 39 minutes, "summer" nerves untreated = 46 minutes, winter nerves plus arsenite = 82 minutes, "summer" nerves plus arsenite = 298 minutes.

3. Sulphydryl mechanisms. According to Voegtlin, Dyer and Leonard

(1923), sulphydryl compounds counteract the toxic effect of a variety of arsenic derivatives on trypanosomes *in vitro* and *in vivo*. It was suggested that the lethal action of arsenite is due to inactivation of intracellular SH compounds by the formation of compounds of the type $R\cdot As(SR)_2$ or $As\,(SR)_3$. Since injection of reduced glutathione does not remove the toxic effect of a previous injection of arsenite, it was assumed that once a reaction has occurred between the fixed SH groups of the tissue proteins, further SH treatment cannot dissociate the compound and the effect is irreversible. Voegtlin, Rosenthal and Johnson (1931) found that whereas reduced glutathione has no stimulating effect on the respiration of tissue cells, it is capable of preventing the arsenic inhibition of respiration when added in a ratio $SH:As = 10$.

A. Action potential. To determine the effect of SH compounds on the arsenite action it was necessary first to test the possible toxicity of these

TABLE 2

Effect of warming frogs (artificial summering) on arsenite extinction of action potential

DATE	DAYS OF PRELIMINARY WARMING	CONCENTRATION OF ARSENITE	TIME FOR EXTINCTION OF ACTION POTENTIAL		REDUCTION OF EXTINCTION TIME
			Warm	Cold	
		M.	*min.*	*min.*	*per cent*
11/18/33	5	0.001	425	565	25
11/18/33	5	0.001	425	565	25
11/27/33	7	0.01	230	681	66
11/27/33	7	0.001	407	787	48
12/ 2/33	12	0.001	307	533	42
12/ 2/33	12	0.01	171	405	58

compounds to nerve. For this purpose the nerves were placed in solutions of cysteine or reduced glutathione[2] in Ringer solution in small stoppered bottles, gently shaken in a thermostat, withdrawn from time to time and the action potentials determined oscillographically. In view of their autoxidizability the solutions were renewed at intervals and frequently tested with nitroprusside. It was found that the spike height was practically unaffected by cysteine or glutathione even in relatively strong solutions. In one experiment M/60 glutathione produced no appreciable decline in six hours.

The next step was to determine whether cysteine or glutathione protects

[2] The cysteine and glutathione solutions were prepared fresh just before each experiment. The weighed solid was dissolved in Ringer solution containing 10 per cent by volume of isotonic sodium phosphate buffer, sufficient M/1 NaOH being added to bring the pH to 7.6–7.8 as determined by the glass electrode. The cysteine was specially prepared to reduce the iron content as described by Warburg.

TABLE 3

Protective action of sulphydryl compounds on the arsenite extinction of the action potential

DATE	TEMPERATURE	CONCENTRATION OF ARSENITE	CONCENTRATION OF SULPHYDRYL		CONCENTRATION RATIO SH/As	EXTINCTION TIME OF ACTION POTENTIAL		INCREASE IN EXTINCTION TIME
			Cysteine	Glutathione		As	As + SH	
	°C.	M.	M.	M.		min.	min.	per cent
8/ 9/33	28	0.001	0.001		1	255	395	55
9/ 8/33	21	0.001		0.01	10	280	367	31
9/11/33	21	0.0001		0.01	100	642	798	24
9/26/33	22	0.0013	0.13		200	230	302	31
10/ 5/33	21	0.001	0.001		1	382	365	−4
10/ 5/33	21	0.001	0.01		10	316	367	16
10/ 5/33	21	0.001	0.033		33	401	505	26
10/ 5/33	21	0.001	0.10		100	406	552	27
11/16/33	23	0.0011	0.06		55	480	720	50
12/29/33	25	0.0017		0.017	10	436	550	26
12/29/33	25	0.0017		0.017	10	230	310	35*

* Nerves were taken from winter frogs which had been kept at 25°C. for 15 days prior to the experiment.

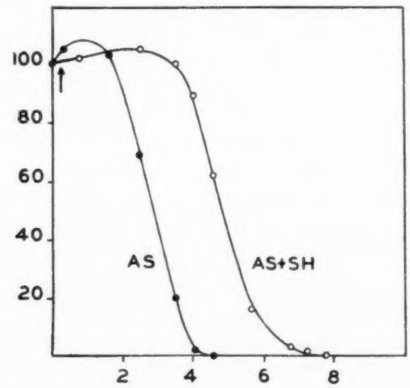

Fig. 1. Cysteine antagonism of arsenite extinction of action potential. Ordinates, action potentials in per cent or original values; abscissae, time in hours. Circles represent effect of 0.005M arsenite containing 0.03M cysteine. Points represent effect of 0.0005M arsenite on partner nerve. Nitroprusside test still positive at end of experiment. Temperature = 20.5°C.

against arsenite. From table 3 and figure 1 it will be seen that such was found to be the case. Whether or not the time to complete extinction is prolonged, SH treatment usually delays the onset of rapid decline of action

potential. In no case, however, did SH prevent the eventual extinction of the action potential regardless of the ratio of SH to As. Nor was it possible, by the addition of SH, to produce any recovery once the action potential had been abolished by arsenite.

B. Respiration. A difficulty in our experiments not encountered by Voegtlin, Rosenthal and Johnson (1931) is the fact that compared with

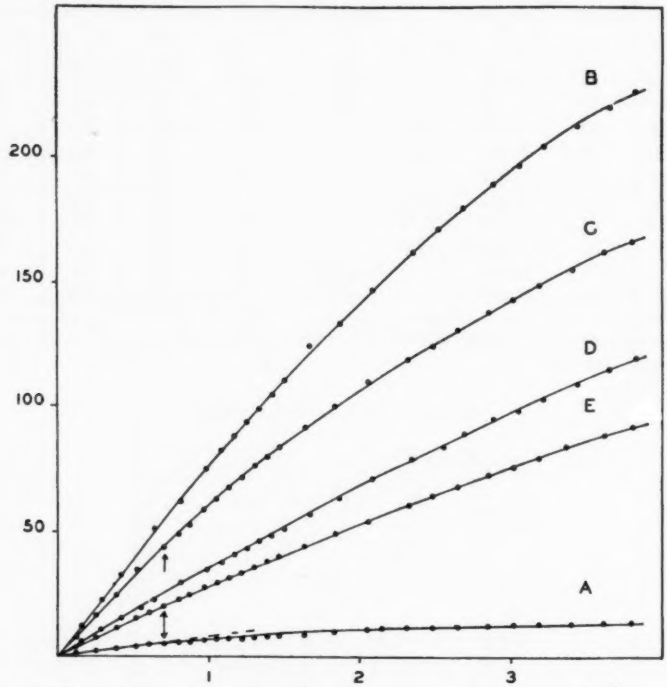

Fig. 2. Effect of cysteine and arsenite on nerve metabolism. Ordinates, oxygen consumption in cubic millimeters; abscissae, time in hours. Final concentrations in all cases: cysteine = 0.133M, arsenite = 0.00084M. Curve *A*, 6 nerves, arsenite added at arrow; curve *B*, 6 nerves plus cysteine; curve *C*, 6 nerves plus cysteine, arsenite added at arrow; curve *D*, cysteine alone; curve *E*, cysteine, arsenite added at arrow. Temp. = 21.7°C., pH = 7.7. Nitroprusside test strong in each vessel at end of experiment. Average action potentials at end of experiment: *A*, 7.1 mm., *B*, 44.6 mm., *C*, 38.1 mm.

the resting oxygen consumption of frog nerve at 20°C., the autoxidation of cysteine or glutathione is relatively high. This makes it difficult to estimate with any exactness the effect of the SH on nerve metabolism. However, in all experiments this autoxidation was allowed for by simultaneous determination of its rate in a separate manometer or by the use of differential manometers in which SH was placed in both vessels.

It was found that nerve, somewhat like kidney in the experiments of Voegtlin, Rosenthal and Johnson, is unable to keep cysteine or glutathione in the reduced form. The nitroprusside test at the end of the experiment was usually either negative or faint whereas that in the vessel containing cysteine alone was still quite positive. Moreover, the oxygen uptake of nerves plus SH was greater than the sum of the uptake of nerves and SH measured alone (see fig. 2). This excess oxygen consumption is probably not due to participation of SH in a catalytic oxidation of nerve, however, for it is not greater than can be accounted for by oxidation of SH to S = S. It appears rather that nerve contains materials which catalyze the autoxidation of SH. While arsenite in concentration ratios, SH:As = 10 to 15, has little inhibiting effect on the autoxidation of SH, it does appear to inhibit the nerve catalysis of oxidation of SH by about 25 per cent or so. In one experiment arsenite was tipped onto the nerves from one side arm first and after the nerve respiration had been reduced by over 60 per cent SH was tipped from a second side arm with the result that the usual increase of oxygen consumption was noted. When SH and As were tipped onto the nerves simultaneously there was a period of an hour or so in which the arsenite inhibition was less than control nerves without SH but in each case typical inhibition soon set in. Despite this failure of SH to protect against arsenite inhibition of respiration, the nerves withdrawn from the vessels at the end of such experiments showed that SH had exerted a decided protective action on the irritable mechanism (see legend, fig. 2).

Discussion. One of the most striking characteristics of the arsenite effect on nerve is the latency of its action. This was noted by Lendle and Reinhardt (1931) and it was shown by Schmitt, Skow and Bueker (1934) that during the induction period the action potential may be practically unaffected although respiration begins to decrease almost at once. While it has been impossible to demonstrate for nerve so close a relationship between arsenite inhibition of respiration and methylene blue reduction as that claimed for muscle by Banga, Schneider and Szent-Györgyi (1931) still a rough relationship has been shown to exist. It is probable, therefore, that the catalytic system paralyzed by arsenite is that which mediates substrate activation. In line with this are the seasonal variations not only in the latency of arsenite action on the irritable mechanism (comparable to the seasonal variation in time to asphyxial block) but also in the methylene blue reduction time. This seasonal variation is due either to variation in the amount or specific kind of substrate present, to a variation in activity of dehydrogenase or to a combination of effects. Collett, Rheinberger and Little (1933), who studied the effect of arsenite on substrate activation in frog muscle, assumed that the poison acts entirely on the enzyme. A seasonal variation in the reduction time of frog muscle was also noted by them but the effect of arsenite on this seasonal variation

appears not to have been studied. Since these authors claim that activation of the various substrates in muscle may be traced by their differential susceptibility to poisoning by arsenite, application of the arsenite inhibition technique may yield clews as to the substrate or group of substrates utilized in nerve activity.

In the absence of reliable quantitative information on the diffusible and fixed SH compounds in nerve it is impossible at the present time to evaluate satisfactorily the rôle of SH in the arsenite effect. Arsenite inhibition of respiration is apparently not prevented to any great extent by preliminary treatment with SH and the temporary protection of the irritable mechanism may be due to the tendency of SH to combine loosely with arsenite, thus decreasing the effective arsenite concentration and delaying action potential block. On the other hand the irreversibility of arsenite action may be explained at least partially by union of arsenite with fixed SH groups in the proteins as suggested by Rosenthal and Voegtlin (1930). Partial denaturation of the proteins exposes SH groups which in turn are muzzled by arsenite. Presumably when this process has involved a certain minimal quantity of structural or enzyme protein, irreversible block occurs.

SUMMARY

1. Arsenite in concentrations sufficient to block nerve action potentials and inhibit respiration 50 to 80 per cent does not inhibit nerve oxidase.

2. Substrate activation as measured by the methylene blue technique is considerably inhibited by arsenite. The degree of inhibition shows marked seasonal variation and the arsenite extinction time of the action potential shows a similar seasonal variation.

3. Although preliminary treatment with cysteine or glutathione has little protective action on the arsenite inhibition of respiration the action potential extinction time may be considerably prolonged thereby. SH compounds are unable, however, to prevent eventual extinction of the action potential or to restore irritability after arsenite block.

4. The induction period during which respiration is inhibited, but action potentials are but little affected, is discussed in terms of the catalytic systems present.

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